BIOTREATMENT OF WATERS CONTAMINATED WITH COMMERCIAL NAPHTHENIC ACIDS

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ABSTRACT

Extraction of bitumen and production of heavy oil from oil sands play a crucial role in the economy of Canada. Processing of oil sands, however, results in the generation of large amounts of contaminated waters (oil sands process-affected water, OSPW) that are currently stored in tailing ponds. These waters and associated tailing ponds are one of health and environmental challenges associated with oil sands; and efforts in the remediation of these contaminated and toxic waters are urgently required. Toxicity of contaminated waters (OSPW) has been attributed mainly to the presence of naphthenic acids (NAs). Different physicochemical and biological approaches have been used for removal of NAs present in the OSPW. These methods include, but are not limited to, adsorption, advanced oxidation, coagulation/flocculation, membrane filtration, biodegradation, and combination of chemical and biological methods. However, much of the research up to now has been focused on the biodegradation pathways, effect of NAs on biodegradation extent, and comparison of different removal methods and little attention has been paid to impact of operating parameters for improving the treatment efficiency. Therefore, this study was set out to explore this missing link.

In the work presented here, an experimental study on biodegradation of NAs was conducted in batch bioreactors and circulating packed bed bioreactors (CPBBs) that aimed to verify the impact of various parameters like initial concentration, temperature, and loading rate on biodegradation of commercial NAs and included evaluation of individual NAs distribution and toxicity. It is expected that understanding of aerobic biodegradation of commercial NAs will lead to the development of suitable technologies to address this environmental concern.

Through the batch experiments, the effect of various parameters including concentration (50, 100, 150, 200 mg NA L^{-1}), and temperature (20, 24, 28, 35 ℃) were studied. Maximum biodegradation rates in the batch system were achieved during the biodegradation of NAs with the highest concentration (0.78 mg TOC L^{-1} h^{-1} at 200 mg TOC L^{-1} and 28 ℃) (TOC: Total organic carbon). It was also observed that temperature variation did not significantly affect the biodegradation of commercial NAs. Gas chromatography-electron impact–mass spectrometry analysis was used to track the changes in NA mixture profiles, or “fingerprints,” for each

condition over time. Using this analysis, more rapid degradation was observed for NAs that had lower carbon numbers and fewer degrees of cyclization.

In continuous packed bed bioreactors (CPBBs), increasing the NA concentration and loading rate led to higher removal rate, while removal percentage was declined. The maximum biodegradation rate of 128.0, 321.7, 430.2, and 630.0 mg TOC L^{-1} h^{-1} and removal percentages of 76.3, 87.6, 89.1, and 82.5% were achieved with influent NA concentrations of 50, 100, 150, and 200 mg NA L^{-1}, respectively. GC-MS compositional study of CPBB effluents showed that low molecular weight NAs were most amenable to biodegradation where for instance at influent concentration of 50 mg NA L^{-1} and loading rate of 7 mg TOC L^{-1} h^{-1} the removal percentage was 83.1, 81.0, and 74.7 for light, medium, and heavy NAs. It was also observed that NAs cyclicity had a direct effect on the removal percentage of NAs. The results of *V. fischeri* and *A. salina* toxicity analyses indicated that biodegradation in CPBB reduced the toxicity of NAs contaminated water. This reduction depended on the loading rate and influent concentration. At influent concentration of 100 to 200 mg NA L^{-1}, IC_{50} of 7.6 and 4.0% improved to 78.1 and 68.5% at the lowest loading rates and 41.7 and 36.5% at the highest loading rates, respectively.

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To my loving ones…

My wife, Nazanin and my daughter, Liana

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LIST OF ABBREVIATIONS

AOP: Advanced oxidation methods

BOD: Biological Oxygen Demand

CF: Coagulation/flocculation

c-NA: Classical Naphthenic Acid

COD: Chemical Oxygen Demand

CPPB: Circulating Packed Bed Bioreactor

CSTR: Continuous Stirred Tank Reactor

EI: Electron Impact

ESI-MS: Electron Spray Ionization- Mass Spectrometry

FBBR: Fluidized Bed Bioreactor

FT-IR: Fourier Transform Infrared Spectroscopy

GC-FID: Gas Chromatography-Flame Ionization Detector

GC-MS: Gas Chromatography-Mass Spectrometry

HPLC-MS: High-Performance Liquid Chromatography- Mass Spectrometry

IC_{50}: 50% Inhibition Concentration

IFAS: Integrated fixed-film activated sludge

ISBR: Immobilized Soil/Sediment Bioreactor

LC_{50}: 50% Lethal Concentration

MBBR: Moving Bed Bioreactor

MBR: Membrane Bioreactor

MFC: Microbial Fuel Cell

MLSB: Mildred Lake Settling Basin

MTBSTFA: N-methyl-N- (tert. -butyldimethylsilyl) trifluoroacetamide

NA: Naphthenic Acid

OA: Octanoic Acid

OSPW: Oil Sands Process-Affected Water

oxy-NA: Oxidized Naphthenic Acid

PAH: Polycyclic Aromatic Hydrocarbons

STP: South Tailing Ponds

t-BDMS: *tert*. -butyldimethylsilyl

TIC: Total Ion Chromatogram

TOC: Total Organic Carbon

WIP: West in-Pit

1. INTRODUCTION

Canada is the fourth largest crude oil producer in the world with 97% of its proven reservoirs as the oil sands (165.4 of 169.9 billion barrels)(National Energy Board, 2017; Natural Resources Canada, 2016). Oil sands production in 2016 was 2.6 million barrels per day accounting for around two-thirds of the total Canadian oil production (National Energy Board, 2017). It is projected that oil sands production will increase by 77% from 2016 to 2040 (National Energy Board, 2017). Extraction of bitumen from oil sands needs large amounts of water (Allen, 2008). In surface mining method, 3 to 4 barrels of fresh water per barrel of bitumen is required, while in the in-situ mining method, this ratio is about 0.4 (Natural Resources Canada, 2016). Oil sands producers recycle around 80-94% of the water used in surface mining and in situ processes (Natural Resources Canada, 2016). Although recycling reduces the need for fresh water, the quality of process water changes and this affects the efficiency of the extraction process. As a result, the extraction process generates contaminated waters, known as OSPW. Due to the zero-discharge legislation passed by the government of Alberta, Canada, OSPW cannot be discharged directly into the environment and it must be stored in tailing ponds for treatment. Today, the volume of tailing ponds in Alberta exceeds 1.18 billion m^3 (in an area of 220 km^2) and it continues to grow (Natural Resources Defense Council, 2017).

Although the composition of OSPW is not the same in all tailing ponds, some contaminants are present in all ponds (Allen, 2008). An important group of contaminants in OSPW are NAs in the form of a complex mixture with a concentration of 20-120 mg L^{-1} (Quagraine *et al.*, 2005). NAs are highly toxic and corrosive, precipitate in pipelines, and reduce the commercial value of petroleum products. Therefore, treatment of NA-contaminated waters is of great importance. Various physical and chemical approaches have been evaluated to address this issue, but cost, safety and implementation on a large-scale can limit their application. Biologically based treatments which exploit the degradative ability of microorganisms are the most cost-effective methods and environmental advantages of them outweigh the alternatives (Scott *et al.*, 2008). Considering that the biological treatment of NAs mixture has not been investigated in detail in some areas, in the current work, this approach will be investigated from

an engineering point of view by focusing the effect of parameters like concentration, residence time, loading rate, and temperature in novel circulating packed-bed bioreactors that are not used for this purpose to date.

The thesis presented here consists of five chapters including introduction, literature review and research objectives, materials and methods, results and discussion, conclusions and recommendations for future work.

2. LITERATURE REVIEW AND RESEARCH OBJECTIVES

2.1. Characteristics of oil sands process-affected waters

The mining of oil sands from deposits depends mainly on the depth of the deposits. For shallow formations of 75 m or less, surface mining is more suitable, while for deeper formations the in situ method is more applicable (Allen, 2008). In surface mining, the Clark Hot Water Process is utilized to extract bitumen from the mined oil sands. Allen (2008) presented a simplified flow diagram of the mining process which shows for oil production of 1,329 t h^{-1}, 6,082 t h^{-1} fresh water is needed and 11,514 t h^{-1} process-affected water is generated. While only 6,364 t h^{-1} of water can be recycled and reused in the process and the remaining must be stored in tailing ponds (Allen, 2008).

Water, dissolved salts, organics, minerals, and bitumen exist in tailing ponds. Mahaffey and Dubé (2017) summarized the average properties of OSPW in different tailing ponds including Mildred Lake Settling Basin (MLSB), West in-Pit (WIP), and South Tailing Ponds (STP) as presented in Table 2.1. These data show the variation of chemical and physical properties of OSPW and factors such as ore quality, source, extraction processes, and OSPW age that affect these properties. However, in general solids (sand, silt, and clays) at 20-30%, and bitumen at 1-3% are the main components of the ponds (Allen, 2008).

Table 2.1 Average properties of oil sands process-affected water (Mahaffey & Dubé, 2017)

Parameter	Active settling basin (Year)		
	Syncrude MLSB (1980-2006) (1980-2006)	Syncrude WIP (1997-2010)	Suncor STP (2008-2010)
pH	8.08	8.35	8.63
Conductivity (μS cm^{-1})	1658	3723	3090
Hardness (mg L^{-1}, $CaCO_3$)	-	93	46
Alkalinity (mg L^{-1}, $CaCO_3$)	542	592	575
Suspended solids (mgL^{-1})	904	828	-
Dissolved solids (mg L^{-1})	1196	242	-
BOD (mg L^{-1})	32	11.9	-
COD (mg L^{-1})	422	226	-
DO (mg L^{-1})	2.3	1.5	-
TOC (mg L^{-1})	45	50	83
NAs (mg L^{-1})	73	70	49

2.2. Naphthenic acids

Hydrocarbon biodegradation in oil deposits leads to the formation of compounds such as acyclic and cyclic, saturated and aromatic carboxylic acids and phenols. NAs is the collective term to describe the complex mixtures of predominantly alkyl-substituted alicyclic carboxylic acids which fit the empirical formula $C_nH_{2n+z}O_xN_\beta S_\gamma$. Where n is the carbon numbers, Z represents the hydrogen deficiency which can be zero or a negative integer, x, is the oxygen atom numbers, indicating whether the compound is classical NA (c-NA; x=2) or oxidized NA (oxy-NA; x≥3), and β and γ are the nitrogen and sulfur numbers, respectively (Ajaero *et al*., 2017; Xue *et al.*, 2018). Most of the NAs found in OSPW have carbon numbers in the range 7 to 30, and Z numbers from 0 to -12 (Whitby, 2010). c-NAs and oxy-NAs with 3 and 4 oxygen atoms are the dominant species in OSPW. Classical species are distinctly the primary group, while oxy- NAs with 3 and 4 oxygen are likely the oxidation products of the classical NAs through biological processes (Xue *et al*., 2018). General chemical and physical properties of c-NAs are summarized in Table 2.2 (Scott *et al*., 2008).

Table 2.2 Physical and chemical properties of c-NAs (Scott, et al., 2008)

Parameters	Properties
Color, Odor, Taste	Pale yellow (dark amber), Musty hydrocarbon odor, Bitter
State, Volatility	Viscous liquid, Non-volatile
Molecular weight	Generally, between 140 and 450 atomic mass units
Solubility	soluble in organic solvents, slightly soluble in water (< 50 mg/L)
Density, pH	0.97–0.99 g/cm^3, 7-8
Boiling point	250–350 ºC
Log (K_{OW})	≈ 4.3 at pH 1.07, 2.38 at pH 7.1 and 2.08 at pH 10
Specific gravity	0.95–0.98 (at 20 ˚C)
Partition coefficient (L kg^{-1})	0.2 for coarse sands and 2.5 for oil sand fines 1.3 for milli-Q water and 17.8 for synthetic groundwater

For the c-NAs, molecular weights differ by 14 atomic mass units (CH_2) between n series and by 2 atomic mass units (2H) between z series (Headley *et al*., 2009; Kannel & Gan, 2012). Because NAs have both carboxylic groups (hydrophilic) and aliphatic groups (hydrophobic), they act as surfactants and are present at aqueous/ nonaqueous interfaces; however, their state in aquatic environments depends strongly on the pH (Kannel & Gan, 2012).

From an industrial point of view, commercial naphthenic acids are used in different applications as wood preservatives, emulsifying agents, paints and coatings. Naphthenic acids are available commercially only as a complex mixture of many different types of compounds. A pure model for naphthenic acids is not available, so surrogate compounds with a similar structure containing a ring with a carboxylated side chain have been extensively used. Nonetheless, because of their simple structure, they cannot be good representatives of OSPW NAs. In addition, OSPW NAs consist of a complex mixture of individual NAs, while considering a surrogate NA, or a mixture of surrogate NAs cannot represent the complexity of real OSPW NAs. To overcome this problem and have a better understanding of OSPW NAs, several biodegradation studies have used commercially available naphthenic acids as surrogates for naphthenic acids found in oil sands wastewaters (Clemente *et al*., 2004; Misiti *et al.*, 2013).

2.3. Toxicity of naphthenic acids

NAs have long been recognized as the primary toxic components of OSPW that show toxicity to a variety of organisms (Clemente & Fedorak, 2005). However, the complex composition of NAs in OSPW makes it difficult to specify the toxic effect of the individual compounds. Other contaminants, like poly aromatic hydrocarbons (PAH) and metals may also contribute to the overall OSPW toxicity. Various organisms' tracts including reproductive, digestive, and respiratory might be impaired by exposure to OSPW (Bartlett *et al*., 2017; Lari *et al*., 2017).

Toxicity effects of OSPW on several types of organisms including prokaryotes (bacteria) and eukaryotes (animals, fish, amphibians, birds, etc.) have been extensively studied and the thresholds are mostly represented in terms of IC_{50}/IC_{20} or LC_{50}. The former is the concentration of a toxic compound causing 50% or 20% inhibition of a specific biological or biochemical function, while the latter is the concentration that kills half of the sample population of a specific test organism over a specific period of time. Li *et al.*, (2017) have summarized the results of studies on the toxicity of OSPW in a recent review with the highlights shown in Table 2.3. This review shows that the sensitivity of organisms to toxic effects of OSPW is variable. For instance, *Daphnia manga* is more susceptible to OSPW than trout and less susceptible than *Vibrio fischeri*. Moreover, source, type, and age of OSPW could be determining factors in its toxicity. The potency of each contaminant including NAs, PAHs, and heavy metals should be specified to develop more efficient methods of OSPW treatment.

Table 2.3 OSPW toxicity in different organisms (Li et al., 2017)

Sample designation	OSPW type	Test organism	Duration of exposure	Endpoint
MLSB	Fresh	*V. fischeri*	15 min	IC_{50}: 32%
OSPW-NAEs	NAs	*V. fischeri*	15 min	IC_{50}: 41-65%
MLSB	Fresh	*D. magna*	96 h	LC_{50}: 16-27%
Recycle water Syncrude	Fresh	*C. dubia*	6-8 days	LC_{50}: 70.7%
MLSB	Fresh	Rainbow trout	96 h	LC_{50} <4%
WIP	Fresh	Rainbow trout	96 h	LC_{25} <25%, LC_{50} <35%

2.4. Treatment of waters contaminated with naphthenic acids

Several methods have been studied for treatment of waters contaminated with NAs. The main features of these methods have been briefly discussed below.

Adsorption has been used to remove contaminants from OSPW. Zubot *et al.*, (2012) found that petroleum coke with the dosage of 30-40% can effectively adsorb NAs with high molecular weight and cyclicity. Similar results have been obtained by other researchers using petroleum coke as an adsorbent (Pourrezaei *et al*., 2014). The reason for effective adsorption of these NA can be due to the higher hydrophobicity and less solubility of NAs with higher carbon and ring numbers. Azad *et al.*, (2013) examined several adsorbents and showed that activated carbon and nickel-based alumina had the highest capacity for NAs removal. These findings also revealed that adsorption occurred very fast at the beginning and the system reached equilibrium conditions quickly. Although some improvements in the activity of adsorbents can lead to higher removal efficiency of NA from OSPW (Niasar *et al.*, 2016), the low adsorption capacity of adsorbents, adsorbent regeneration costs, as well as the generation of a secondary pollutant (contaminated adsorbents when regeneration is costly) all limit the application of this method.

Photo-catalysis is based on the reduction and oxidation reactions that take place due to the formation of electron-hole pairs on the surface of the catalyst. An important characteristic of this method is the adsorption of the contaminants onto the photon activated catalyst surface. Studies showed that TiO_2 has the most favorable properties as a photo-catalyst. Headley *et al.*, (2009) investigated the photodegradation of OSPW NAs, commercial and surrogate NAs and reported that TiO_2 in the presence of sunlight removed 75% of NAs even in OSPW and that sunlight was more efficient than fluorescence light. Mishra *et al.*, (2010) and Leshuk *et al.*, (2016) found the source of NAs, type of water, the use of TiO_2 catalyst, and dissolved oxygen can significantly change the photodegradation rate of NAs, while temperature and pH had less impact. A recent study showed the modification of catalyst surface structure enhances photocatalytic activity (Liu *et al.*, 2016). One of the drawbacks associated with the photo-catalysis is the difficulty in separating the TiO_2 catalyst after treatment, limiting its use on an industrial scale. Another challenge is the development of a catalyst with a broader photo-activity range and its feasible integration into a reactor system.

Membrane filtration is a broad term describing a process wherein a fluid is passed through a porous immobilized solid material, allowing for the removal of select solutes from the fluid stream. Polymeric, ceramic, and micellar-enhanced membranes have been extensively used for the treatment of waters contaminated with NAs. Micellar-enhanced ultrafiltration can remove up to 98% of surrogate NAs present in water (Deriszadeh *et al.*, 2009; Husein *et al.*, 2011; Venkataganesh *et al.*, 2012). The composition of polysulfone and ceramic membranes with carbon nanotubes, as well as pretreatment of OSPW with coagulants can improve the NAs removal efficiency (Kim *et al.*, 2013; Alpatova *et al.*, 2014; Benally *et al.*, 2018). Membrane separation can also be integrated with biological treatment in membrane bioreactors to enhance the removal rate of NAs (Xue *et al.*, 2016). However, fouling not only reduces permeate flux but may also cause reductions in the removal efficiency of the membrane. Therefore, to maintain a constant effluent flow rate under fouling conditions, the trans-membrane pressure must be increased and that in turn leads to an increase in operation cost.

Coagulation/flocculation (CF) involves promoting the collision of small particles to form larger particles, which may then be easily separated from the solution by either flotation or sedimentation. Utilization of polyaluminum chloride as a coagulant did not affect the removal of NAs. It, however, led to a reduction in the toxicity of OSPW by removing metals like Fe, Al, Ga, and Ti (Wang *et al.*, 2015). Although many recent studies have reported CF can only remove fine solids in OSPW (Wang *et al.*, 2015; Lu *et al.*, 2016; Thompson *et al.*, 2017; Vajihinejad & Soares , 2018), it has also been shown it can be an effective method for NA removal. Using this approach, Pourrezaei *et al.*, (2011) were able to remove 37% of c-NAs and 86% and oxy-NAs in OSPW by the use of alum and a cationic polymer. While CF processes are often energy efficient and simple to maintain, high chemical consumption and sludge production are some of the limitations associated with these processes (Quinlan & Tam, 2015).

Ozonation is a clean and efficient approach for the breakdown of organic pollutants, with ozone playing the role of the oxidant and precursor of other oxidizing radicals. Scott *et al.*, (2008) found ozone is an effective oxidizer for OSPW treatment. In their research, the concentration of NAs in the OSPW was reduced, while total organic carbon (TOC) remained constant. They suggested that NAs were not completely mineralized to CO_2 but oxidized to other intermediate organics that remained in solution. The GC-MS analysis of ozone treated samples after 130 min showed more than 90% of NAs with carbon number higher than 22 were degraded.

Therefore, most of the recalcitrant NAs were broken down and the bio-availability of NAs in OSPW increased. Reduction in the toxicity of OSPW was another noticeable outcome. Cost and safety issues associated with utilization of ozone, and generating some toxic oxy compounds are some concerns in using ozonation.

Advanced oxidation methods (AOPs) is a broad term for processes in which chemical compounds are partially or completely degraded through oxidation reactions with hydroxyl radicals. These hydroxyl radicals are often produced in situ by the activation of hydrogen peroxide using some stimulus, typically a transition metal catalyst, UV light, or ozone. The oxidation of NAs proceeds through a complex network of multiple series-parallel reactions involving radical species derived from the oxidizing agents as well as from the organic substrate and the intermediates in the reaction pathway. Four advanced oxidation processes (UV/TiO_2, UV/IO_4^-, $UV/S_2O_8^{2-}$, and UV/H_2O_2) were studied by Liang *et al.*, (2011) to investigate their ability to mineralize NAs to inorganic carbon in OSPW. The final concentration of 5 mg L^{-1} NAs was achieved using $UV/S_2O_8^{2-}$ and UV/H_2O_2 at pH 8-10, with the required electrical energy for UV/H_2O_2 being much less than $UV/S_2O_8^{2-}$. UV/Chlorine (Chan *et al.*, 2012; Shu *et al.*, 2014), UV/Fenton (Zhang *et.al*, , 2017), and several chemical oxidizers (Sohrabi *et al.*, 2013; Wang *et al.*, 2016) were also able to remove NAs and attenuate OSPW toxicity. The principal drawbacks of traditional AOPs for full-scale applications are the relatively high capital and operating costs for implementation and the high level of energy needed for these processes.

Bioremediation of NAs has been investigated to assess the rate of biodegradation and the effect of structural and operating parameters. NAs used in these investigations fall into three categories: surrogate NAs; commercially available NAs; and NAs in OSPW. Often, the goal of the investigations that used surrogate NAs was to elucidate the biodegradation pathways of the individual NAs. For example, these studies report that the addition of methyl groups hinders NA biodegradation; and trans- isomers are more amenable to biodegradation than their cis-geometric isomers (Headley *et al.*, 2002). Generally, due to the lower molecular weight of the model and commercial NAs, they are more readily degraded than the OSPW NAs. It appears that OSPW NAs, particularly the recalcitrant NAs, have structures consisting of branched alkyl chains which affect biodegradation rates (Han *et al.*, 2008). In addition to the chemical structure of NAs, other factors such as the surfactant properties of the NAs and their associated toxicity in aquatic

environments may also cause resistance to microbial biodegradation. Thus, bioremediation of NA-contaminated waters will require microorganisms capable of oxidizing such compounds with branched alkyl groups and thrive in the complex and toxic environment associated with NAs.

On the other hand, investigations that used actual naphthenic acids (commercial NAs or OSPW NAs) focused on the biodegradation of these compounds as a group, because current analytical methods do not allow the study of individual compounds in the complex mixture. Some of these investigations monitored the decrease in toxicity as the naphthenic acids were biodegraded. Biodegradation especially in batch mode is a very slow process, taking months or even years to degrade some recalcitrant species. So, it requires to combine with other remediation methods to expedite the process.

Integrated methods use at least two methods of degradation of NAs. Integration of biodegradation as the most cost-effective treatment option for treatment of OSPW with other methods can remove recalcitrant NAs efficiently. Various studies have been carried out to investigate the relationship between the effectiveness of biodegradation as a result of pre-treatment by advanced oxidation processes. Ozonation is usually used in combination with biological treatment to reduce costs. Generally, ozonation can be applied first at a relatively low concentration of ozone, which can break recalcitrant organic compounds. By-products of ozonation may be still toxic or even more toxic than the original compounds if not fully mineralized. Therefore, the subsequent biological treatment is required to remove some of the residual organic compounds formed during the ozonation process. The integrated ozonation and biological treatment may provide a viable option to remove contaminants from OSPW economically and effectively (Hwang *et al*., 2013; Islam *et al*., 2014).

2.5. Bioreactors

One of the most common approaches in ex-situ bioremediation is the use of bioreactors. The function of the fermenter or bioreactor is to provide a suitable environment in which an organism can efficiently produce a target product—the target product might be cell biomass, metabolite and bioconversion product. It must be so designed that it is able to provide the optimum environments or conditions that will allow supporting the growth of the microorganisms. The design and mode of operation of a fermenter mainly depends on the production organism, the optimal operating condition required for target product formation,

product value and scale of production. Bioreactors exist in a variety of configurations and various modes of operations. Configurations of bioreactors include continuous stirred tanks bioreactors, bubble column bioreactors, airlift bioreactors, fluidized bed bioreactors, packed bed reactors, and membrane bioreactors (Nemati & Webb, 2011).

One of the most conventional bioreactors is the stirred-tank bioreactor. The core component of the stirred tank bioreactor is the agitator or impeller, which performs a wide range of functions: heat and mass transfer, aeration, and mixing for homogenization. There are many advantages of STRs over other types. These include the efficient gas transfer to growing cells, good mixing of the contents and flexible operating conditions, besides the commercial availability of the bioreactors (Wang & Zhong, 2007).

In the bubble column bioreactor, the air or gas is introduced at the base of the column through perforated pipes or plates. The flow rate of the air/gas influences the performance factors including mass transfer and mixing. The simple construction of bubble-column reactors makes them easy to maintain. In addition, it is possible to control the degree of shear, uniformly within the reactor, which is critical to the growth of plant and animal cells in particular (Kadic & Heindel, 2014).

In the airlift bioreactors, the medium of the vessel is divided into two interconnected zones by means of a baffle or draft tube. In one of the two zones referred to a riser, the air/gas is pumped. The other zone that receives no gas is the down comer. The dispersion flows up the riser zone while the down flow occurs in the down comer. There are two types of airlift bioreactors. Internal-loop airlift bioreactor has a single container with a central draft tube that creates interior liquid circulation channels. These bioreactors are simple in design, with volume and circulation at a fixed rate for fermentation. External loop airlift bioreactor possesses an external loop so that the liquid circulates through separate independent channels. These reactors can be suitably modified to suit the requirements of different fermentations. In general, the airlift bioreactors are more efficient than bubble columns, particularly for more denser suspensions of microorganisms. This is mainly because in these bioreactors, the mixing of the contents is better compared to bubble columns (Wang & Zhong, 2007).

Fluidized bed bioreactor is comparable to bubble column bioreactor except the top position is expanded to reduce the velocity of the fluid. The design of the fluidized bioreactors (expanded top and narrow reaction column) is such that the solids are retained in the reactor

while the liquid flows out. These bioreactors are suitable for use to carry out reactions involving fluid suspended biocatalysts such as immobilized enzymes, immobilized cells, and microbial flocs (Godia & Sola, 2005).

A bed of solid particles, with biocatalysts on or within the matrix of solids, packed in a column constitutes a packed bed bioreactor. A nutrient broth flows continuously over the immobilized biocatalyst. The products obtained in the packed bed bioreactor are released into the fluid and removed. While the flow of the fluid can be upward or downward. Because of poor mixing, it is rather difficult to control the pH of packed bed bioreactors by the addition of acid or alkali. However, these bioreactors are preferred for bioprocessing technology involving product-inhibited reactions. The packed bed bioreactors do not allow accumulation of the products to any significant extent (Warnock & Bratch, 2005).

Membrane Bioreactors (MBRs) can be broadly defined as systems integrating biological degradation of waste products with membrane filtration. They have proven quite effective in removing organic and inorganic contaminants as well as biological entities from wastewater. Advantages of the MBR include good control of biological activity, high quality effluent free of bacteria and pathogens, smaller plant size, and higher organic loading rates. Current applications include water recycling in buildings, wastewater treatment for small communities, industrial wastewater treatment, and landfill leachate treatment (Iorhemen *et al.*, 2016).

Circulating packed bed bioreactor is a combination of external loop airlift bioreactor and packed bed bioreactor. The packing material is put in the riser section. The improved mass transfer rate can provide sufficient oxygen for microorganism attached on packing material to degrade the targeted organic contaminants in an efficient manner. The packing material also provides a stable surface for microbes to attach to, as a consequence, biomass hold-up in the bioreactor is also improved (Huang *et al.,* 2012).

2.6. Identification of naphthenic acids

Identifying the NAs helps to track their variation upon treatment. Currently, there is no universal established method to quantify individual NAs and most of the current analytical methods treat NAs as a group, or as sub-groups based on carbon and Z numbers. Fourier transform infrared spectroscopy (FTIR) was among the first methods for measuring the NAs. However, methods based on mass spectrometry (MS) have been playing a prominent role in

analyses of NAs present in aqueous solutions. Because NAs are a complex mixture of carboxylic acids, they can be extracted from an acidic aqueous solution into an organic solvent or from petroleum into an alkaline aqueous solution. These extraction methods are often used in preparation for quantitative or qualitative analyses of NAs. Briefly, these methods are as follows:

1. FTIR: the absorbances of the monomeric and dimeric forms of the carboxylic groups are measured and compared to those in a calibration curve obtained from the FTIR analyses of solutions prepared with a commercially available NAs (Holowenko *et al.*, 2001; Barrow *et al.*, 2004; Scott *et al.*, 2008). FTIR serves to determine not only NAs but also a total signal of various detectable characteristic bonds that may not correspond to NAs. So, it shows higher concentration of NAs compared to MS techniques.
2. GC-FID: the derivatized form of NAs are analyzed using GC with flame ionization detection. The result is an unresolved hump that is integrated and compared to the area of internal standard (Paslawski *et al.*, 2009).
3. ESI-MS: MS with negative or positive ion ESI has been used to determine NAs concentrations in aqueous samples. Concentrated NAs from aqueous samples by solid phase extraction, were analyzed by ESI-MS (Dias *et al.*, 2014; Wang *et al.*, 2016).
4. HPLC-MS: derivatized products of NAs are injected into an HPLC column. Calibration curves are prepared by integrating the humps produced from the analyses of derivatized commercial NAs preparations (Afzal *et al.*, 2015; Hindle *et al.*, 2013; Shang *et al.*, 2013).
5. GC-MS: this is the most common method in laboratories, which does not require sophisticated analytical equipment. After GC analysis of derivatized NAs to their t-butyldimethylsilyl esters, the average mass spectrum of the unresolved hump is taken, and the ions are assigned to the appropriate carbon and Z numbers. These data are used to determine the relative abundance of acids in the NAs mixture (Merlin *et al.*, 2007; Folwell *et al.*, 2016).

2.7. Knowledge gap and research objectives

Bioremediation studies aiming at the treatment of NAs have been conducted generally in the microcosms and small-scale culture systems. Furthermore, the focus of these studies has been mainly on the biodegradability of NAs, impacts of molecular structure on the extent of biodegradation, and finally identification of biodegradation pathways. Past research in our group has focused on the aerobic biodegradation of individual and mixture of surrogate NAs in various

bioreactor configurations such as continuous stirred tank bioreactors (CSTR), packed-bed, and CPBB (D'Souza *et al.*, 2014; Huang *et al.*, 2012), as well as anaerobic biodegradation of NAs, coupled to the reduction of nitrate and nitrite (Gunawan *et al*., 2014). Another focal point of earlier research in our group has been the utilization of MFC bioreactors that allows the treatment of contaminated waters with the concomitant production of energy. Building on the existing knowledge, the work has expanded to study treatment of waters contaminated with commercial NAs. Given the complicated composition of OSPW NAs and the presence of the recalcitrant NAs in the mixture, commercial NAs that contain a variety of NAs with different molecular structures were used as a surrogate to study the biotreatment of NA-contaminated waters. The specific objectives of this research were as follows:

1. to study the aerobic biodegradation kinetics for commercial NAs including the effects of concentration, and temperature in batch systems;
2. to evaluate the aerobic biodegradation of commercial NAs in CPBBs including the effect of concentration and loading rate of NAs; and
3. to assess the toxicity of raw and treated effluents obtained under various operating conditions and in different treatment systems.

3. MATERIALS AND METHODS

Experimental works in this research have been divided into two parts: batch experiments aiming to assess biodegradation of commercial NAs, and continuous biodegradation of waters contaminated with commercial NAs. The details of each part including materials used and procedures are described in the following sections.

3.1. Materials

A mixture of NAs from Tokyo Chemical Industries (Sodium Naphthenate, CAS RN: 61790-13-4; Tokyo Chemical Industries Co., Ltd., Japan) was used in all biodegradation experiments. These will be referred to as commercial NAs hereafter. Octanoic acid ($C_8H_{16}O_2$, CAS RN: 124-07-2; Alfa Aesar) was used as the surrogate NA for microbial culturing and development of biofilm in CPBBs. *Artemia Salina* (San Francisco Bay Brand, Inc. Newark, CA, USA) were used for the toxicological studies.

3.2. Microbial culture and medium

Aerobic mixed culture originated from the soil of an industrial site contaminated with heavy hydrocarbons was used as the initial culture (Paslawski *et al.*, 2009). Previously, different surrogate NAs have been used for acclimation of this culture. The compositional analysis of this culture had led to the identification of *Pseudomonas aeruginosa* and *Variovorax paradoxus* as the dominant species (Paslawski *et al.*, 2009). These species reportedly have the potency to remove recalcitrant hydrocarbon compounds of wastewater.

In current work, microbial culturing was started using octanoic acid as the substrate, which was then gradually substituted by commercial NA to ensure bacteria were acclimated to the new substrate and they were able to digest commercial NAs. Cultures were maintained in 250 ml flasks each containing 200 ml of modified McKinney's medium with 100 mg NA L^{-1} octanoic acid or commercial NAs. To start, octanoic acid (OA) was dissolved in the medium and pH was adjusted between 7.0 and 7.2. The medium was then inoculated with 20 ml of the 7 days old culture (10% v/v). Sub-culturing was carried out every week. After several sub-culturing with OA, commercial NAs was gradually added to the substrate. This started using a mixture of

90% OA and 10 % of commercial NAs. It was then slowly shifted to lower concentrations of OA and higher commercial NAs (e.g. 80 and 20%, 60 and 40%, 40 and 60%, 20 and 80% OA and commercial NAs, respectively). Each step was cultured few times and ultimately the pure commercial NAs solution was used as a substrate.

Modified McKinney's medium was used for culture maintenance and in biodegradation experiments (Gunawan *et al*., 2014; Paslawski *et al*., 2009). This medium provides the necessary nutrients for culture growth and biodegradation experiments. Modified McKinney's medium was prepared using reverse osmosis water (RO water) and had the following composition: KH_2PO_4 (840 mg L^{-1}), K_2HPO_4 (750 mg L^{-1}), $(NH_4)_2SO_4$ (474 mg L^{-1}), NaCl (60 mg L^{-1}), $CaCl_2$ (60 mg L^{-1}), $MgSO_4.7H_2O$ (60 mg L^{-1}) and Fe $(NH_4)_2(SO_4)_2.6H_2O$ (20 mg L^{-1}). Trace solution which is containing nutritional requirements of cells was added to the modified McKinney's medium at 0.1% (v/v). The composition of trace solution is as follows: H_3BO_3 (600 mg L^{-1}), $CoCl_3$ (400 mg L^{-1}), $ZnSO_4.7H_2O$ (200 mg L^{-1}), $MnCl_2$ (60 mg L^{-1}), $NaMoO_4.2H_2O$ (60 mg L^{-1}), $NiCl_2$ (40 mg L^{-1}) and $CuCl_2$ (20 mg L^{-1}). The medium is autoclaved for 15 minutes at 121 °C prior to use.

3.3. Experimental Setup

3.3.1. Batch experiments

Batch experiments were conducted to study biodegradation of commercial NAs under aerobic conditions. The effects of commercial NA concentration and temperature were investigated in 250 ml Erlenmeyer (shake) flasks as batch reactors. Different initial concentrations of commercial NAs (50, 75, 100, and 200 mg NA L^{-1}) were evaluated in duplicate. Each flask was inoculated with a 7-day old culture (10% v/v). Flasks were maintained at room temperature (24 ± 2°C) on a shaker. Samples were taken regularly and analyzed for NAs concentration and molecular weight distribution. The temperature controlled environmental chamber was utilized to assess the effect of temperature in the range of 20-35 °C. Because of the chamber's limitations, it was not possible to consider the effect of temperature at lower temperatures (5-20 °C). At each temperature, various concentrations of commercial NAs were evaluated (50-200 mg L^{-1}). Control experiments were conducted under similar conditions without inoculation.

3.3.2. Specification of Circulating Packed Bed Bioreactors (CPBB)

Continuous biodegradation of commercial NAs was studied under aerobic conditions in two CPBBs, which are made of transparent glass (Figure 3.1). The total volume of CPBBs were 562 mL and 468 mL. The bioreactors had inlet and outlet ports, which allowed continuous operation of the bioreactors. Stainless steel mesh (80% porosity) was used as a carrier matrix for the establishment of biofilm.

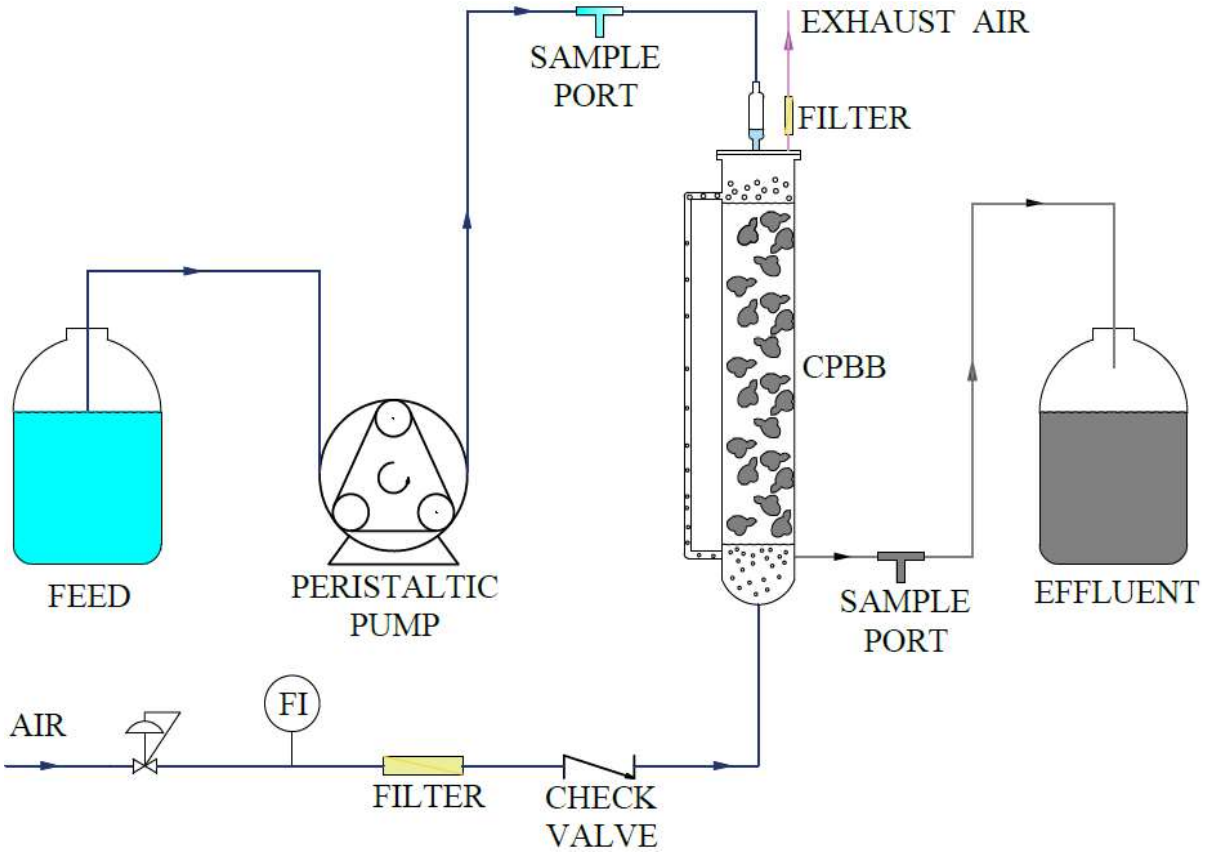


Figure 3.1 Schematic flow diagram of a typical circulating packed bed bioreactor and other components of the experimental setup

In each bioreactor, the effluent was removed through an overflow tube. The effluent port was elevated to maintain sufficient liquid in the bioreactor. Air was introduced into the reactor from the bottom through a flow meter. The air flow rate was maintained at 0.3-0.5 $Lmin^{-1}$ (Huang *et al*., 2012). The bioreactor columns, tubes, as well as feed containers, were sterilized

using both bleach and ethanol prior to conducting the experiments. The CPBBs specifications are listed in Table 3.1.

Table 3.1 Specification of the circulating packed bed bioreactor (CPBB)

Parameter	1st CPBB	2nd CPBB
Riser height (cm)	35	31
Riser diameter (cm)	4.5	4.1
Downcomer height (cm)	32	29
Downcomer diameter(cm)	0.5	0.5
Packing Porosity	0.8	0.8
Working volume* (ml)	350	375
Total reactor volume (without packing) (ml)	562	468

* The working volume was determined at the end of experiments with biofilm attached to the support.

3.3.3. Development of Biofilm

The biofilm in the bioreactor developed over a period of 12 weeks, where sterile modified McKinney's medium containing 500 mg NA L^{-1} octanoic acid and 10% (v/v) inoculum was trickled at a flow rate of 10-40 ml h^{-1}. Simultaneously, partially degraded effluent was recycled back into the reactor at a rate of 2.4 L h^{-1}, which provided a continuous source of viable microorganisms and substrate. Figure 3.2 shows the bioreactor setup and the developed biofilm at the early and final stages.

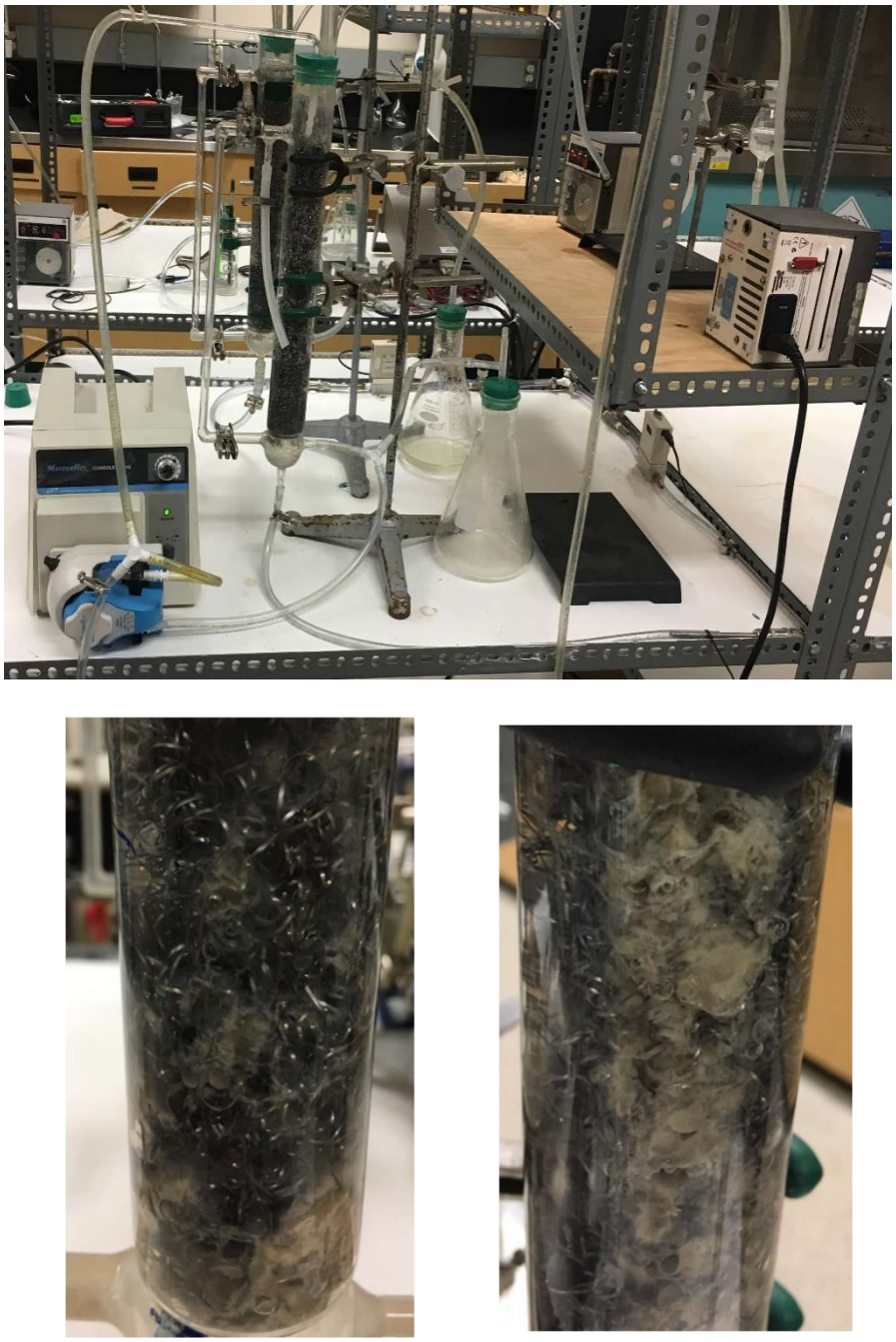


Figure 3.2 Photos of bioreactor set-up and developed biofilm in the CPBB.

(top: bioreactor setup, bottom: biofilm development at early and final stages)

3.3.4. Procedure for continuous experiments in CPBBs

Upon the formation of biofilm in the bioreactors which could be seen easily, the main experiments were initiated by draining the reactor and supplying it with sterile modified McKinney's medium containing commercial NA using a peristaltic pump that introduced the feed to the upper part of the bioreactor. Simultaneously, the effluent was removed from the bottom of the bioreactor through the overflow tube. Effects of commercial NAs concentration (50, 100, 150, and 200 mg NA L^{-1}) and loading rate were investigated by applying a wide range of flow rates (85-2200 ml h^{-1}). The flow rate was set using a calibrated pump and was measured on a regular basis by weighing the effluent collected over a specified period. At each flow rate, enough time was given for the establishment of steady-state conditions, which was verified by stability in the residual substrate concentrations (variation less than 10%).

Furthermore, to assess the reproducibility of the experimental results, duplicate experiments were conducted in a number of flow rates at the end of the experiments. All the experiments were performed at room temperature (24±2°C).

Bioreactor effluent was sampled regularly to determine the concentration and molecular weight distribution of NAs, pH, and toxicity. The composition of the developed biofilm was analyzed at the end of experimental runs using an external laboratory. The generated effluent and all the collected samples were sterilized and disposed of according to appropriate protocols as outlined in the University of Saskatchewan Safety Resources Policy (2018). The same procedure was applied to the bioreactor liquid contents upon completion of each experimental run.

3.4. Analytical Methods

3.4.1. Total Organic Carbon (TOC)

A total organic carbon analyzer was used to determine the organic carbon content of the influent and the treated effluent (TOC-L Shimadzu, TOC-LCPH, Canada). The TOC analyzer used catalytic oxidation method at 680°C and had a detection limit of 4 µg TOC L^{-1}. Before TOC analysis, bacteria were removed from the sample by centrifuging 15 ml of the effluent sample at 14000 rpm (18000 g) using a microfuge (Microfuge@ 18 Centrifuge, BECKMAN COULTER TM) for 6 min. This was followed by filtering 10 ml of supernatant through a 0.22 µm

pore diameter nylon membrane syringe filter. The influent sample was used directly for analysis. The TOC analyzer had a built-in system for the removal of inorganic and purgeable carbon.

3.4.2. Analysis of Naphthenic Acids by GC-MS

Gas chromatography-electron impact ionization mass spectrometry (GC-EI-MS) was used to determine the molecular weight distribution of naphthenic acids in the influent and effluent. Electron impact mass spectrometry (EI-MS) provides some information about NA structure and impurities. However, due to the extensive fragmentation of aliphatic molecules in the mass spectrometer, limited molecular mass and structural information can be obtained about NA components. To minimize the effect fragmentation St. John *et al.* (1998) method was used. The technique is based on the analysis of *tert*. -butyldimethylsilyl (t-BDMS) derivatives of NA. The t-BDMS derivative was obtained by reaction of *N*-methyl-*N*- (*tert*. -butyldimethylsilyl) trifluoroacetamide with the acidic proton on alcohols or carboxylic acids. The derivative is very stable and has been used to enhance resolution in some difficult gas chromatography analyses. Additionally, the water in the samples should be eliminated to have more concentrated NAs and to prevent column bleeding. Therefore, before analysis pretreatment of samples was required to assure water was completely separated and minimum fragmentation would occur in the mass spectrometer. This pretreatment includes separation of bacteria by centrifugation and filtration, extraction by solvent, and derivatization as described below.

First, 15 ml of sample was centrifuged at 14000 rpm for 6 min followed by filtration of 10 ml of supernatant through a 0.22 μm pore diameter nylon membrane syringe filter. This was then acidified to pH < 2 using 2 M sulfuric acid (H_2SO_4, CAS: 7664-93-9; GR ACS). This solution was extracted twice with dichloromethane as solvent (CH_2Cl_2, CAS RN:75-09-2; Sigma Aldrich) in a 1:1 by volume of sample to solvent ratio. The resulting extracts were combined, and the solvent was evaporated to a volume of 1 ml using a rotary evaporator (Buchi, Rotavapor R300). NAs in the concentrated solution was derivatized using N-methyl-N-(tert. -butyldimethylsilyl) trifluoroacetamide (MTBSTFA) which contained 1% tert.-butyldimethylsilylchloride ($C_9H_{18}F_3NOSi$, CAS RN:77377-52-7; Sigma Aldrich). In brief, derivatizations were performed by adding 100 μl of the MTBSTFA reagent to 100 μl of extracted NA. Samples were then heated to 60℃ for 20 min to generate the t-BDMS derivative (St.John *et al.*, 1998).

The GC-MS analysis was performed using an Agilent GC (7890A)- MS (5975C) equipped with an Agilent J&W HP-5MS (Nonpolar, (5%-Phenyl)-methylpolysiloxane) capillary column (30 m × 0.25 mm, 0.25 μm film thickness, 7-inch cage). The injection was run in splitless mode. The helium carrier gas flow rate was set at 1 ml min^{-1}. The inlet temperature was kept at 280 ℃ to ensure everything would be vaporized. The GC and MS interface temperature was set at 300 °C. The oven initial temperature was held at 70 °C for 3 min before ramping at a rate of 8 °C min^{-1} to a maximum temperature of 300 °C that was held for 5 min. The solvent delay was set at 8 min. The GC-MS was operated in electron impact ionization mode with ion source temperature and quadrupole temperature at 230 and 150 ℃, respectively. The MS was set to scan for m/z ratio of 157 to 550 with a scan rate of 5 scans per second. An injection volume of 2 μL was chosen to ensure that saturation would not occur (i.e., no m/z peak could exceed 10^7 counts). Every three samples, pure solvent was run with the same method as a blank. Chromatograms and spectra were acquired and analyzed using the Agilent MSD ChemStation program.

Peak ion intensity values were averaged over the elution of the naphthenic acids hump, generally from retention time 10 min and higher. The ‘‘minimum occurrence’’ variable for the averaged data was set at 1%, which meant that the peak ion had to occur in at least 1% of the total scans to be included in the final average data output from the computer (St.John *et al.*, 1998). The m/z values obtained were used to determine the NAs molecular weight, carbon number, and hydrogen deficiency (Z) with the detailed procedure described in Appendix A.

3.4.3. Toxicity tests

Toxicity of influents with different NA concentrations and effluents obtained under various conditions were evaluated using brine shrimp (*Artemia salina*) and Microtox@ methods. Brine shrimp toxicity tests were carried out for all influents and treated effluents obtained in the continuous CPBBs under various operating conditions. Given the associated costs and extended time required, Microtox@ was done on selected samples representing important operating conditions.

The influent solution was obtained by solving commercial NAs in McKinney’s medium. In the case of effluents before toxicity test, bacteria were removed using the exact procedure used for the preparation of samples before TOC analysis (Section 3.5.1).

The procedure for brine shrimp toxicity test was similar to the procedure described by Anhaecke *et al.*, (1981). First, 100 mg of brine shrimp eggs were added to a cylindrical tube containing 100 ml of tap water and aerated for one hour. Then 3.5 g of NaCl was added to maintain a salt concentration of 35 g L^{-1}. The tube was kept at room temperature and was aerated for another 24 hours to allow shrimps to hatch. The tube was then laterally illuminated for 5 min to allow unhatched eggs to come to the surface and hatched shrimps sink to the bottom. Then, concentrated hatched shrimps were sucked out by a pipette and transferred to a flask containing salt water. This was followed by transferring 0.5 ml aliquot of this solution that contained approximately 20 shrimps into each of four watch glasses. For the first watch glass, 2 ml of McKinney's medium was added as the control sample. The other three watch glasses received 2 ml of influent or effluent samples (i.e., the toxicity test of each sample was carried out in triplicates). The watch glasses were monitored for the number of live (motile) shrimps. A 7X magnification Optivisor headset was used to count all motile shrimps. Counting was carried out at times 0, 1, 2, and 4 hours with time zero representing the time when samples were added to watch glasses.

Before the brine shrimp test, the stored samples (treated effluents) were re-analyzed by TOC and the obtained results were compared with those obtained with fresh samples during the experiments in CPBBs to ensure the substrates were not degraded during the storage.

Microtox$^{@}$ test was conducted by an external laboratory (ALS Environmental Lab in Winnipeg, MB, Canada).

3.4.4. Microbial Community Analysis

Analysis of the microbial community was done at the end of experimental runs using an external laboratory (Contango Strategies Ltd. in Saskatoon, SK, Canada). For this purpose, a biofilm sample collected from CPPB operated with commercial NAs was used. The MoBIO PowerLyzer PowerSoil DNA Isolation Kit was used and sequence analysis of the 16S ribosomal RNA (rRNA) was carried out (Labrada & Nemati, 2018).

3.5. Statistical Analysis

For continuous experiments in CPBBs, the system was maintained at each flow rate for a minimum of three residence times after the establishment of steady state. At least three samples

were taken during this period. The data obtained from these samples were used to calculate the average values and associated standard deviations. The standard deviations were calculated using Microsoft Excel and presented as error bars. In addition, the reproducibility of the experimental data was assessed by running the bioreactor at some influent concentrations and loading rates for an extended period with repeated sampling as stated previously.

4. RESULTS AND DISCUSSION

This chapter presents the results of experimental work aiming at the removal of commercial naphthenic acids from contaminated waters. This is started with the results of commercial NAs batch biodegradation and is followed by the results of continuous biodegradation of commercial NAs in circulating packed bed bioreactors (CPBBs).

4.1. Batch biodegradation of naphthenic acids

4.1.1. Effect of initial concentration

Batch biodegradation experiments were carried out to evaluate the effects of NAs initial concentration and temperature. A wide range of NA initial concentrations (50, 100, 150, and 200 mg NA L^{-1}) was examined to verify the potential of microbial culture in biodegradation of commercial NAS. Effect of concentration was investigated at four temperatures (20, 24, 28, and 35 °C) to verify the effect of temperature on the biodegradation of NAs. The results are presented in terms of change in total organic carbon concentration and molecular weight distribution of NAs. Profiles of biodegradation of commercial NAs at different initial concentration are shown in Figure 4.1.

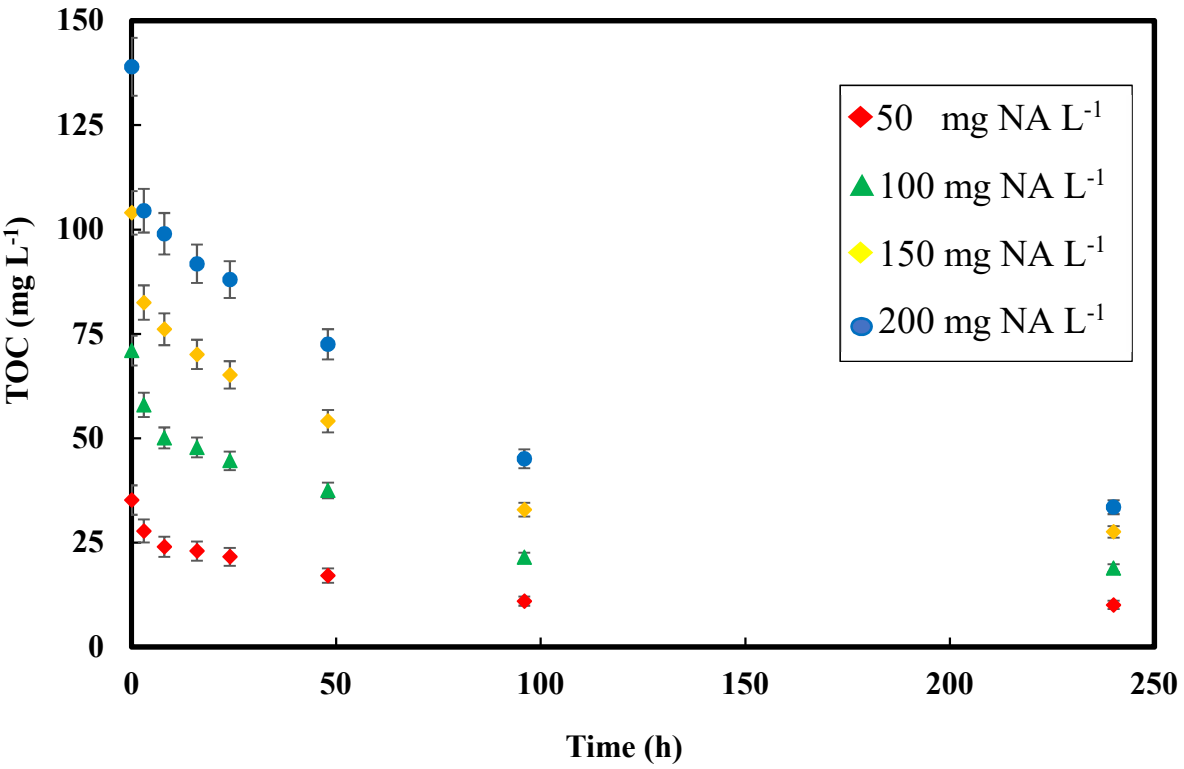


Figure 4.1 Variation of TOC concentration with time obtained with various initial concentrations of commercial NAs in batch cultures at room temperature. The error bars represent the standard deviations of data.

Figure 4.1 shows that TOC decreased sharply in the first day and about 40% of TOC was consumed in 24 hours. This could be explained by the fact that bacterial cells started to grow quickly; and as a result, commercial NAs concentration declined. However, the exact reason for this initial sharp decrease is not clear. This could be potentially attributed to a physical phenomenon such as adsorption of NAs by bacterial cells. After a day, however, this trend changed to slower biodegradation rates. Approximately, 50% and 70 % of total organic carbon were converted by day 2 and 4, and after 10 days, about 25% of TOC remained unchanged. It is now established from a variety of studies that most of these organic carbons were mineralized to CO_2 (Herman *et al.*, 1994; Headley *et al.*, 2002; Clemente *et al.*, 2004). However, in a recent study, Misiti *et al.* (2013) indicated that although a large portion of the commercial NA mixture was biotransformed, only a small fraction was mineralized to CO_2. The non-mineralized NAs were likely biotransformed into more oxidized metabolites. Data in Figure 4.1 also illustrates

that the culture had enough potential to remove the high concentration of commercial NAs (200 mg NA L^{-1}) which is 2-3 times higher than the NA concentration observed in the tailing ponds. In the control experiments where commercial NAs at different concentration were used without any inoculum (sterile conditions) TOC concentration were constant over the 10 days of incubation as shown in Figure 4.2.

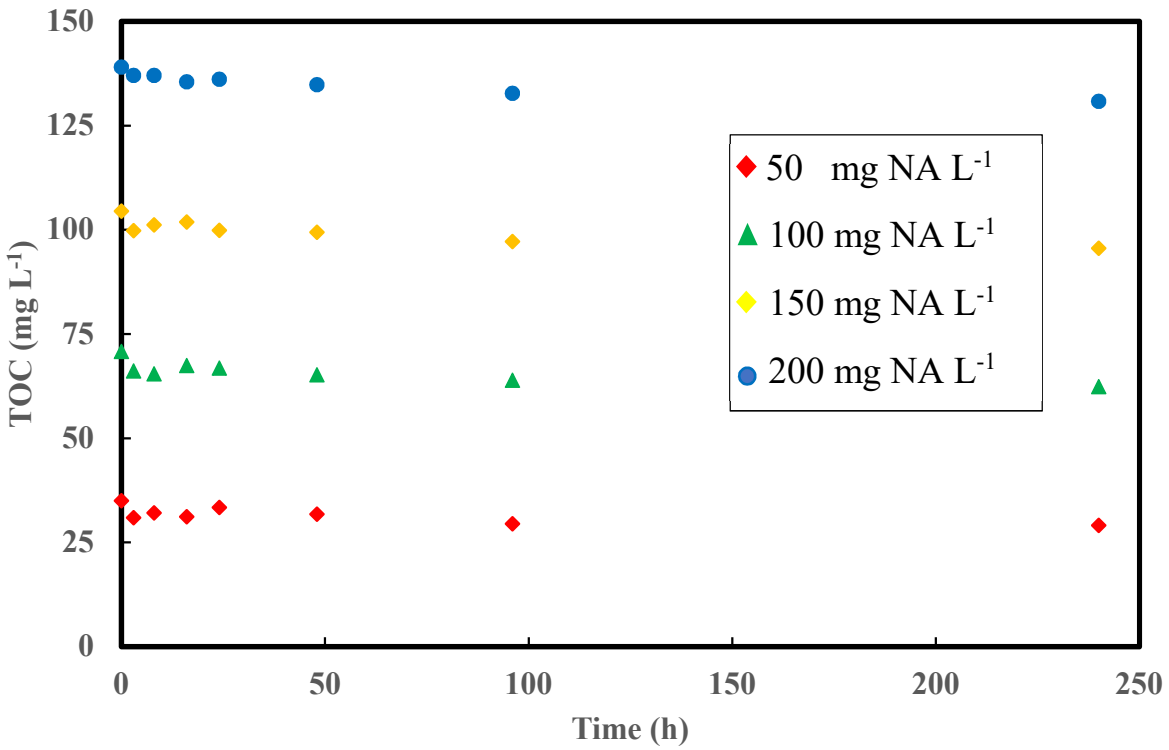


Figure 4.2 Variation of TOC concentration in control experiments at various commercial NAs initial concentrations.

Using the concentration change in the first 4 days of experiments (linear portion of TOC concentration profiles) biodegradation rates were determined and presented in Table 4.1.

Table 4.1 Effect of commercial NAs initial concentration on biodegradation rate

Initial concentration (mg NA L^{-1})	Biodegradation rate (mg TOC L^{-1} h^{-1})	R^2
50	0.20 (0.81)	0.81
100	0.42 (0.84)	0.84
150	0.60 (0.84)	0.84
200	0.76 (0.80)	0.80

From Table 4.1, a marked increase in biodegradation rates can be seen when the initial NAs concentration was increased from 50 to 200 mg NA L^{-1}. This finding is in agreement with the fact that the rate is proportional to the concentration. Previously for the batch biodegradation of surrogate NAs (100 mg NA L^{-1}), the biodegradation rate of 1.1-1.2 mg NA L^{-1} h^{-1} had been reported (Paslawski *et al.*, 2009; Huang *et al.*, 2012; Labrada & Nemati, 2018). Compared to them, the obtained results for the biodegradation of commercial NAs showed slower rates. This decrease further supports that biodegradability is decreased with increase in complexity of NAs structure. Overall, it can be concluded that the biodegradation rate of commercial NAs is strongly influenced by its initial concentration (Huang *et al*., 2012). It is possible that total NAs concentrations would have continued to decrease if the study had been allowed to continue for longer than 10 days.

It had been planned to utilize ultraviolet (UV) spectrophotometry to determine the optical density as an indicator of biomass concentration, but this analysis proved to be challenging in practice. The major problem with this analysis was that after dissolving commercial NAs in McKinney's medium, a change in turbidity of the solution was observed. This change in turbidity then impacted the measured optical density due to microbial activity and led to an error source in estimating biomass concentration. To date, there is no study which has been reported the biomass concentration during biological treatment of OSPW or commercial NAs. This can be due to similar observations.

GC-MS analyses of culture samples were carried out to determine the distribution of naphthenic acids in the original NAs mixture and the culture at different stages of biodegradation experiments and the total ion chromatogram (TIC) is presented in Figure 4.3.

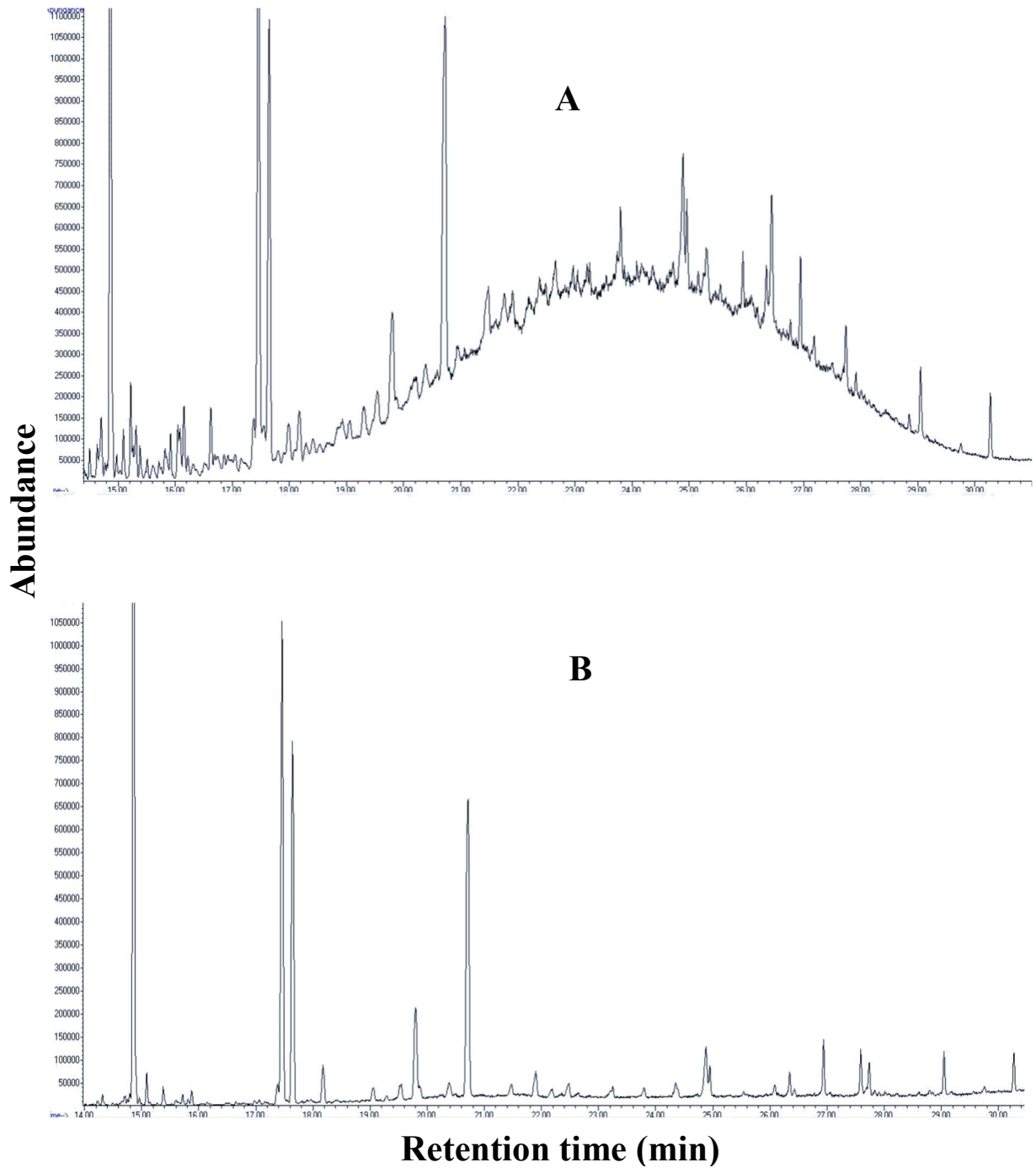


Figure 4.3 Total ion chromatograms of t-BDMS derivatized commercial NA in the original mixture (A) and the culture after 10 days of biodegradation (B).

As seen in Figure 4.3, the total ion chromatogram (TIC) of the original NAs mixture appeared as a hump of unresolved compounds, with several peaks protruding from the hump as observed in other studies (St John *et al.*, 1998; Holowenko *et al.*, 2002; Clemente *et al.*, 2003; Scott *et al.*, 2005). The TIC chromatogram of the NAs in the culture that had been incubated for 10 days showed only a handful of peaks.

Selected results from GC-MS analyses are shown as three-dimensional graphs in Figure 4.4. The graph shows the abundance of ions as a function of n and Z values (carbon and hydrogen deficiency numbers). These ions are a result of NAs ionization in mass spectrometry so that each ion is consistent with an individual NA. Each single bar in the three-dimensional graph represents the sum of different isomers of an individual naphthenic acid in the mixture that corresponds to a specific carbon number and specific Z family, corresponding to specific *m/z* values (molecular weight) from GC–MS analysis.

According to literature, the majority of the commercial naphthenic acids have carbon number n <23, which by considering the general formula of NAs ($C_nH_{2n+z}O_2$), molecular weights of these commercial NAs fall below 330 (Vaiopoulou *et al.*, 2015; Bertheussen *et al*., 2018). As can be seen in Figure 4.4 panel A, the commercial NA used in all experiments are mostly dominated by 0, 1, 2, and 3 ring NA structures (Z= 0, -2, -4, -6) with a significant number of aliphatic compounds present in carbon number range of 13-21 so that the three-dimensional plots in all GC-MS figures were truncated at carbon number 28.

Taking into account this typical commercial NA structure, it is possible to make some assumptions and estimate the number of carbons present within a NA of known molecular weight (e.g., current commercial NAs). Assuming the presence of one carboxyl moiety per NA, 32 Da is accounted for by the presence of two oxygen atoms. Another assumption is that the majority of carbon atoms are bonded to two hydrogen atoms. Therefore, each CH_2 segment accounts for 14 Da. For each additional ring within the structure, there are two fewer hydrogen atoms (2 Da) present per ring. Regarding these assumptions, commercial NA has a weighted average molecular weight (MW) of 235.5 Da and the average formula of $C_{14.8}H_{25.9}O_2$ which are consistent with reported data of Misiti *et al.* (2013). They also showed that the commercial NA is a representative of the types of NAs found in refinery wastewater streams.

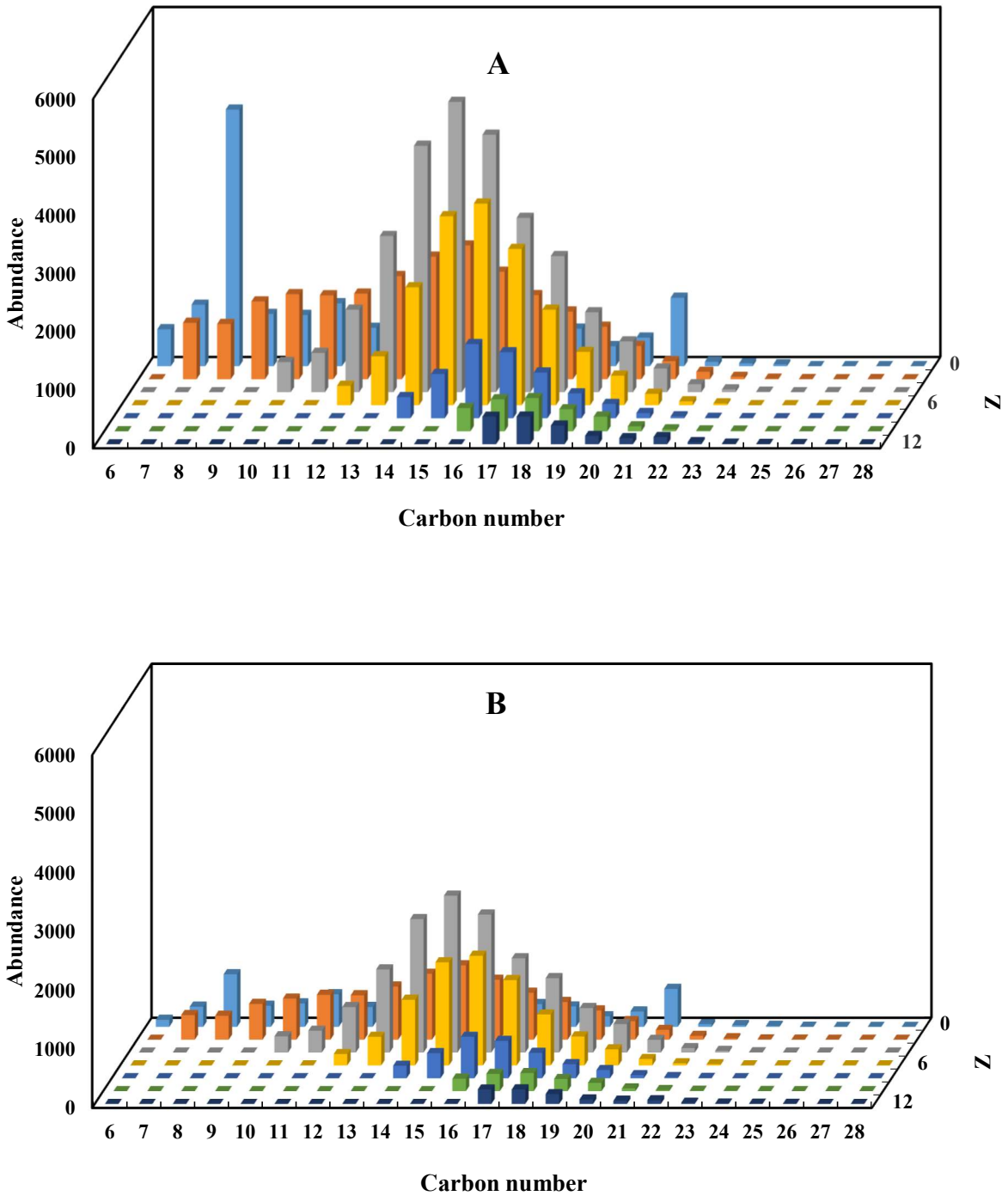
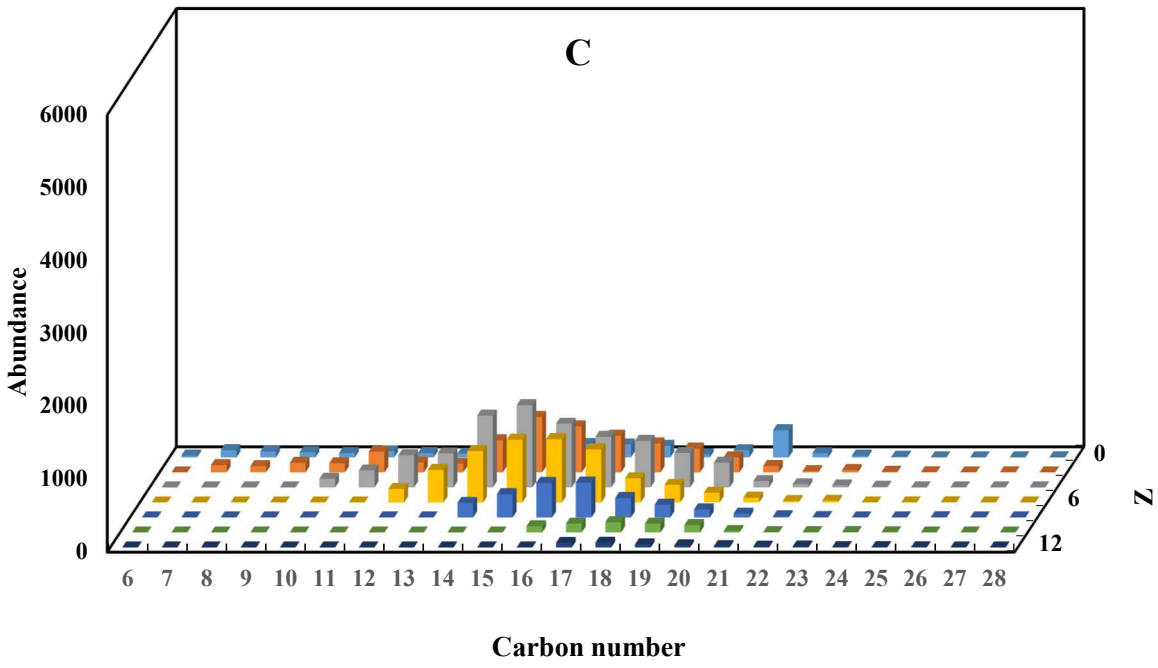


Figure 4.4 The distribution of naphthenic acids with the initial concentration of 50 mg NA L^{-1} in McKinney's medium before biodegradation (A), Four days biodegradation (B), Ten days biodegradation (C)

Figure 4.4 Continued



The NA distribution profile of untreated influent samples of various initial concentration was similar to profiles in the literature obtained by GC–MS (Clemente *et al.*, 2004; Han *et al.*, 2008; Bertheussen *et al.*, 2018). According to Figure 4.4 A for the distribution of NAs in the substrate, it can be seen that the most abundant ion had *m*/*z* of 295. This corresponds to the major fragment ion of the derivative of NAs with n= 15, and Z= -4 (i.e., $C_{15}H_{26}O_2$), comprising 5.4% of all the NAs represented in the bar graph which is quite similar to the results reported for Merichem and Kodak NAs (Clemente *et al.*, 2004).

Compared with NAs original mixture that had a wide array of naphthenic acids (Figure 4.4A), there were virtually small residual naphthenic acids in the sample from the culture that underwent 10 days of biodegradation (Figure 4.4C). So that there was a remarkable change in the fingerprint of naphthenic acids. It means that most individual NAs were consumed and only a few of them were able to resist against the bacterial activity. A similar fingerprint was observed for days between (Figure 4.4B). Fingerprinting studies are contributing to a better understanding of the individual naphthenic acids.

Clemente *et al*. (2004) described a statistical method to compare the fingerprints of NAs mixture, based on the carbon number distribution. As part of their method, the NAs were categorized into three groups: (1): C_5-C_{13}, (2): C_{14}- C_{21}, and (3): C_{22}^+ cluster. The same method was used to group and sum the ion abundances in each GC-MS analysis of the original substrate mixture and samples undergone biodegradation. According to data presented in Figure 4.4A, it was calculated that initially (original substrate) 29% of the ions had masses that fell into the range of the C_5-C_{13} NAs and 70% of the ions fell into the range of the C_{14}-C_{21} NAs. This calculation was performed through a MATLAB® code, which is discussed in detail in Appendix A. The proportion of ions in the first group dropped to 24% at the end of day 4. In the meantime, it was 74 and 2% for the C_{14}- C_{21} and C_{22}^+ groups, respectively. A further change in the percentage of NAs groups occurred, and by day 10, 17% of the ions fell into the range of C_5-C_{13} NAs, 82% of the ions fell into the range of the C_{14}-C_{21} NAs. This examination showed that low molecular weight NAs were most susceptible to biodegradation. By decreasing the percentage of low molecular weight NAs, the relative proportion of other groups increased. However, due to the complexity of the commercial NAs, the mechanism by which this occurs is not well understood, and these statistical methods can only assist in categorizing the complex mixture of NAs.

Hydrogen deficiency number (Z) is another important factor to study the distribution of NAs. From Figure 4.4A and based on Z number, the calculated percentages of NAs in substrate were 18.1, 22.8, 30.6, 18.1, 5.8, 2.6, and 1.9% for Z= 0, -2, -4, -6, -8, -10, -12, respectively. After 4 days of biodegradation, this distribution changed to 14.3, 23.1, 32.2, 19.2, 6.4, 2.8, and 2% (Figure 4.4B) and at day 10, the final distribution was 9.6, 21.4, 32, 22.5, 10.2, 3, and 1.3% (Figure 4.4C). These results show that finding a trend for the effect of Z number on NAs removal is quite difficult. Though, a noticeable decline in the NAs with Z=0 from 18.1% to 9.6% suggests that NAs without cyclicity are the most amenable to biodegradation compared to the monocyclic NAs, bicyclic NAs, and NAs with higher cyclicities.

Cultures developed with higher NAs initial concentration were also analyzed by GC-MS, and the obtained results for the highest NAs initial concentration (200 mg NA L^{-1}) are presented in Figure 4.5.

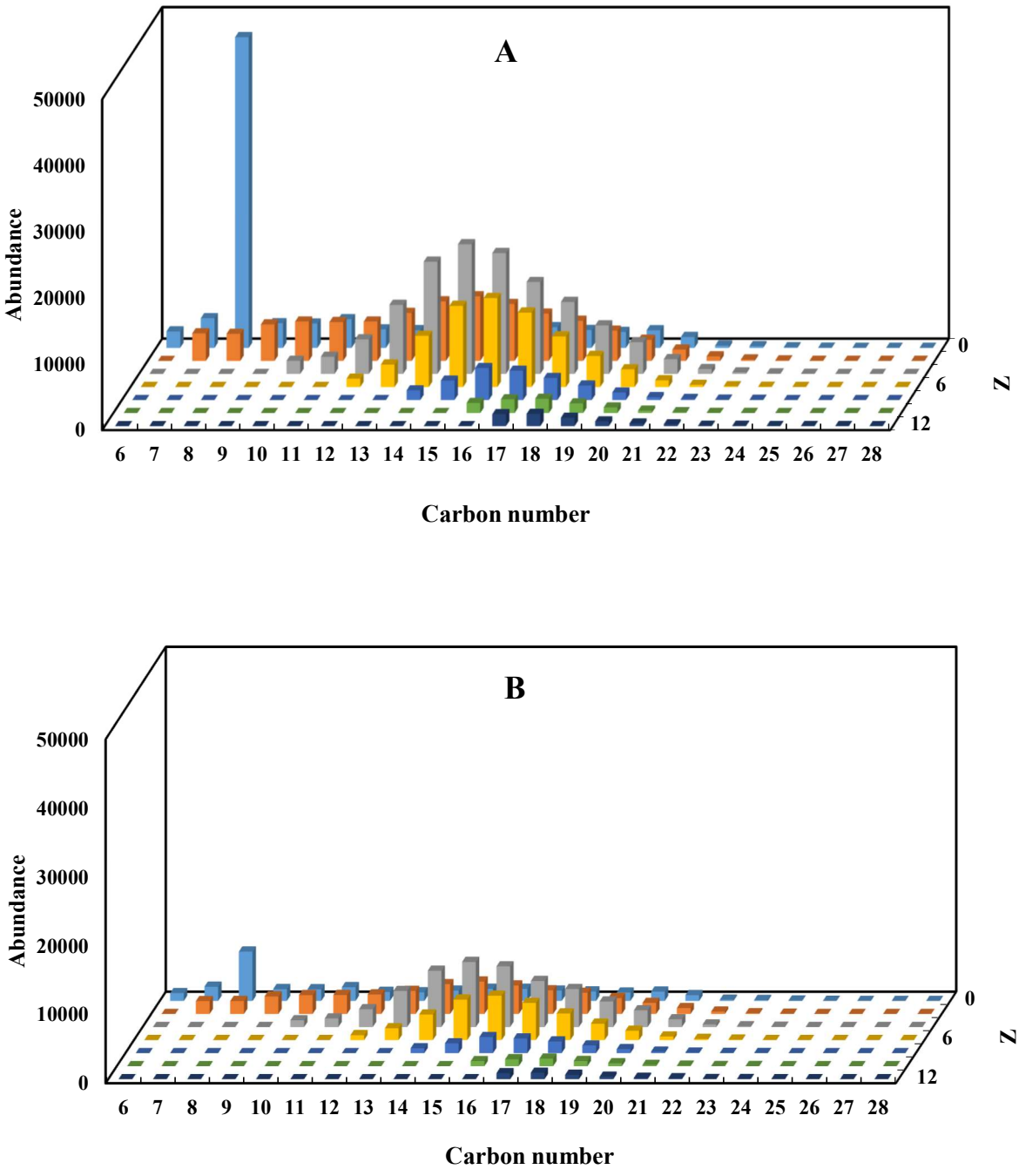
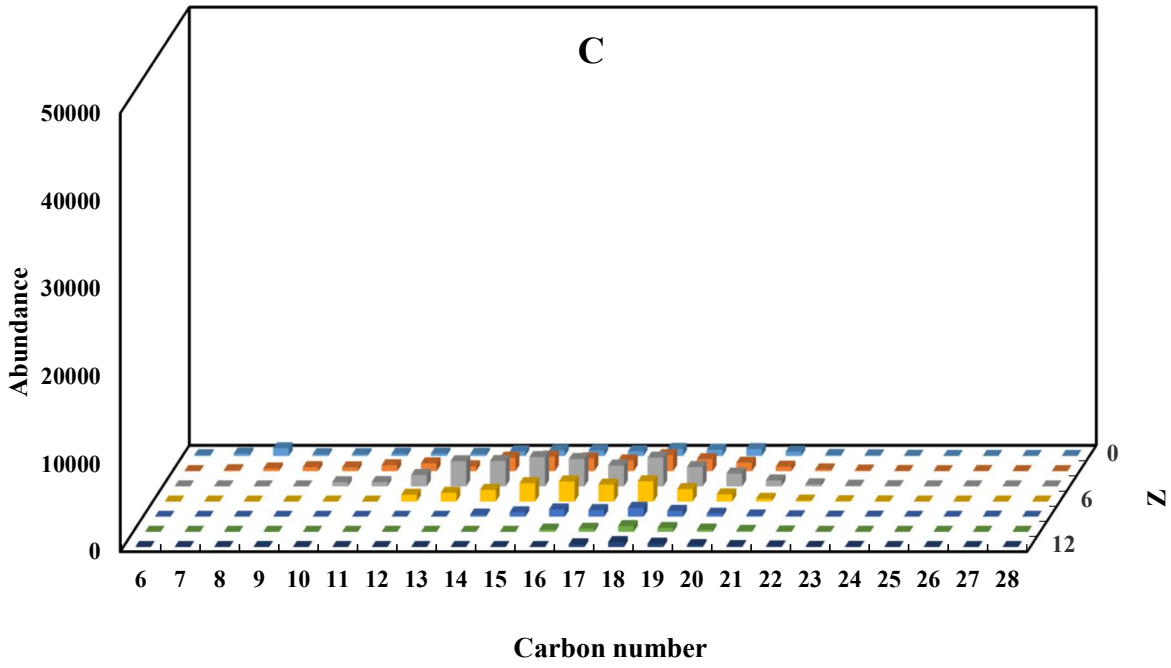


Figure 4.5 The distribution of naphthenic acids with the initial concentration of 200 mg NA L^{-1} in McKinney's medium before biodegradation (A), Four days biodegradation (B), Ten days biodegradation (C)

Figure 4.5 Continued



From panel A, it is clear that 33.5% and 65.4 % of the ions were in C_5-C_{13} and C_{14}-C_{21} groups, respectively. While after 4 days, NAs in the first group decreased to 27.1% and consequently, the percentage of the second group increased to 71.7% and by continuing biodegradation to day 10, 18.3% and 79.8% of the ions fell into the C_5-C_{13} and C_{14}-C_{21} groups. Panels A-C of this figure also reveal that NAs with Z=0 have the highest removal (24% to 11%) compared to other Z families.

By comparing the GC-MS results of cultures with lowest (50 mg NA L^{-1}) and highest (200 mg NA L^{-1}) NAs initial concentration (Figure 4.4and Figure 4.5), a general trend in biodegradation pattern of NAs can be seen. The developed bacterial culture has more potential to degrade NAs with lower molecular weight and less cyclicity even for higher initial concentration. However, increasing the initial concentration caused more NAs to remain unchanged after 10 days.

A number of studies have examined biodegradation of commercial NAs in batch mode. Han *et al.*, (2008) found that only 1.7% of the Refined Merichem NAs remained after 28 days of incubation at room temperature. At the day 28, they could only detect polycyclic NAs while NAs

with Z=0 and -2 totally disappeared. Clemente *et al.* (2004), examined the biodegradation of two commercial NAs. For Kodak NAs after 10 days of incubation, the NAs concentrations dropped from about 86 to less 8 mg NA L^{-1}. They found a similar rate for biodegradation of Merichem in which initial NAs concentration of 109 dropped to 8 at the end of day 10. This was accompanied by the release of about 60% of the carbon from the NAs as CO_2. This biodegradation also led to the formation of oleic, linoleic, palmitic and stearic acids. GC-MS results demonstrated that biodegradation changes the composition of the complex mixture of these NAs and that the lower molecular weight acids (C_5 to C_{13}) were degraded more readily than the high molecular weight acids. In another study, Biryukova *et al.*(2007) investigated the ability of microorganisms enriched from the rhizospheres to degrade commercial NAs. Their results showed a 90% decrease after 10 days of incubation in viable cultures. Lower molecular mass naphthenic acids were preferentially degraded, while the proportion of high molecular mass acids increased during incubation.

4.1.2. Effect of Temperature

The effect of temperature on the biodegradation of commercial NAs was also examined because temperature is considered one of the important parameters affecting the microbial activities and biodegradation process. These experiments were carried out at four different temperatures ranging from 20 to 35°C, with the initial commercial NAs concentrations of 50 to 200 mg NA L^{-1}. Due to limitations of the existing temperature-controlled chamber, conducting experiments at lower temperatures was not possible. Profiles of TOC concentrations obtained in these experiments are shown in

Figure **4.6**.

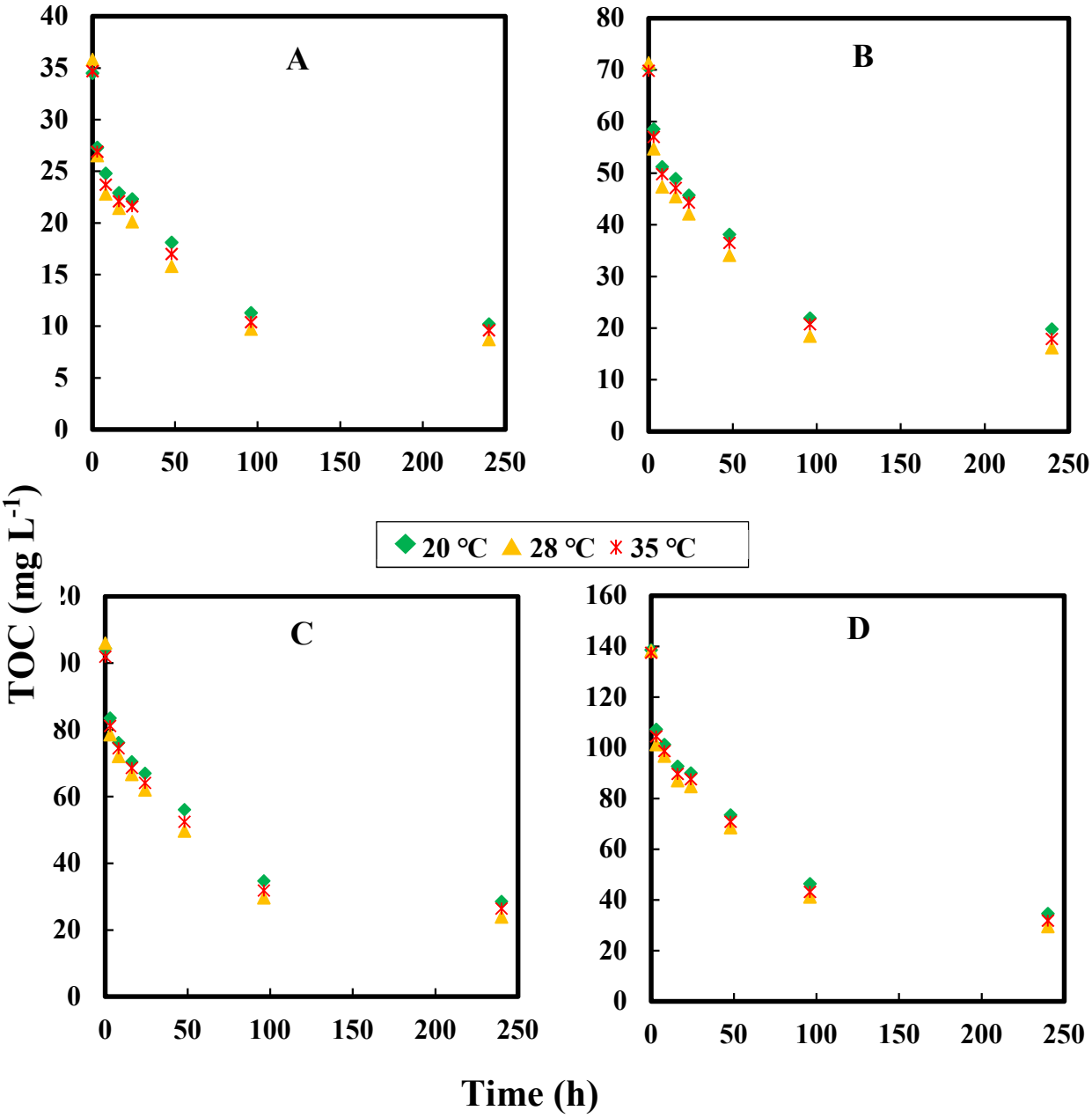


Figure 4.6 Variation of TOC in batch cultures at different temperatures obtained with various initial concentrations of commercial NAs prepared in McKinney's medium: 50 mg NA L^{-1} (A), 100 mg NA L^{-1} (B), 150 mg NA L^{-1} (C), 200 mg NA L^{-1} (D).

As seen in

Figure **4.6**, increasing temperature from 20 ℃ to 24 ℃ and then to 28 ℃ led to a minor lower final residual TOCs, while further increasing the temperature caused a slight increase in the residual TOC. So, temperature variation in the range of 20- 35 ℃ cannot be a detrimental factor on biodegradation of commercial NAs because as the temperature increased nor bacterial growth neither death rate changed significantly. Therefore, the concentration of viable cells and residual TOC remained unchanged. Using the linear portion of profiles (first four days) the biodegradation rates were calculated at different temperatures and are presented in Table 4.2.

Table 4.2 Effect of temperature on biodegradation rate (mg TOC L^{-1} h^{-1}) of commercial NAs [1].

Initial concentration	**Biodegradation rate (mg TOC L^{-1} h^{-1})**			
(mg NA L^{-1})	**20 ℃**	**24 ℃**	**28 ℃**	**35 ℃**
50	0.19 (0.83)	0.20 (0.81)	0.21 (0.75)	0.20 (0.81)
100	0.42 (0.87)	0.42 (0.84)	0.44 (0.82)	0.42 (0.86)
150	0.59 (0.84)	0.60 (0.84)	0.62 (0.79)	0.60 (0.84)
200	0.76 (0.83)	0.76 (0.80)	0.78 (0.80)	0.77 (0.82)

[1] The numbers in the parentheses represent R^2 values

This data revealed that at each initial concentration, temperature variation did not affect the biodegradation rate of commercial NAs. Data from this table can also show that biodegradation rate is mostly influenced by concentration than temperature.

In order to assess the effect of temperature on the distribution of NAs, the cultures that underwent 10 days biodegradation were analyzed by GC-MS and the selected results at an initial concentration of 100 mg NA L^{-1} for Z=0 (most amenable to biodegradation) and Z= -4 (most abundant) are presented in Figure 4.7.

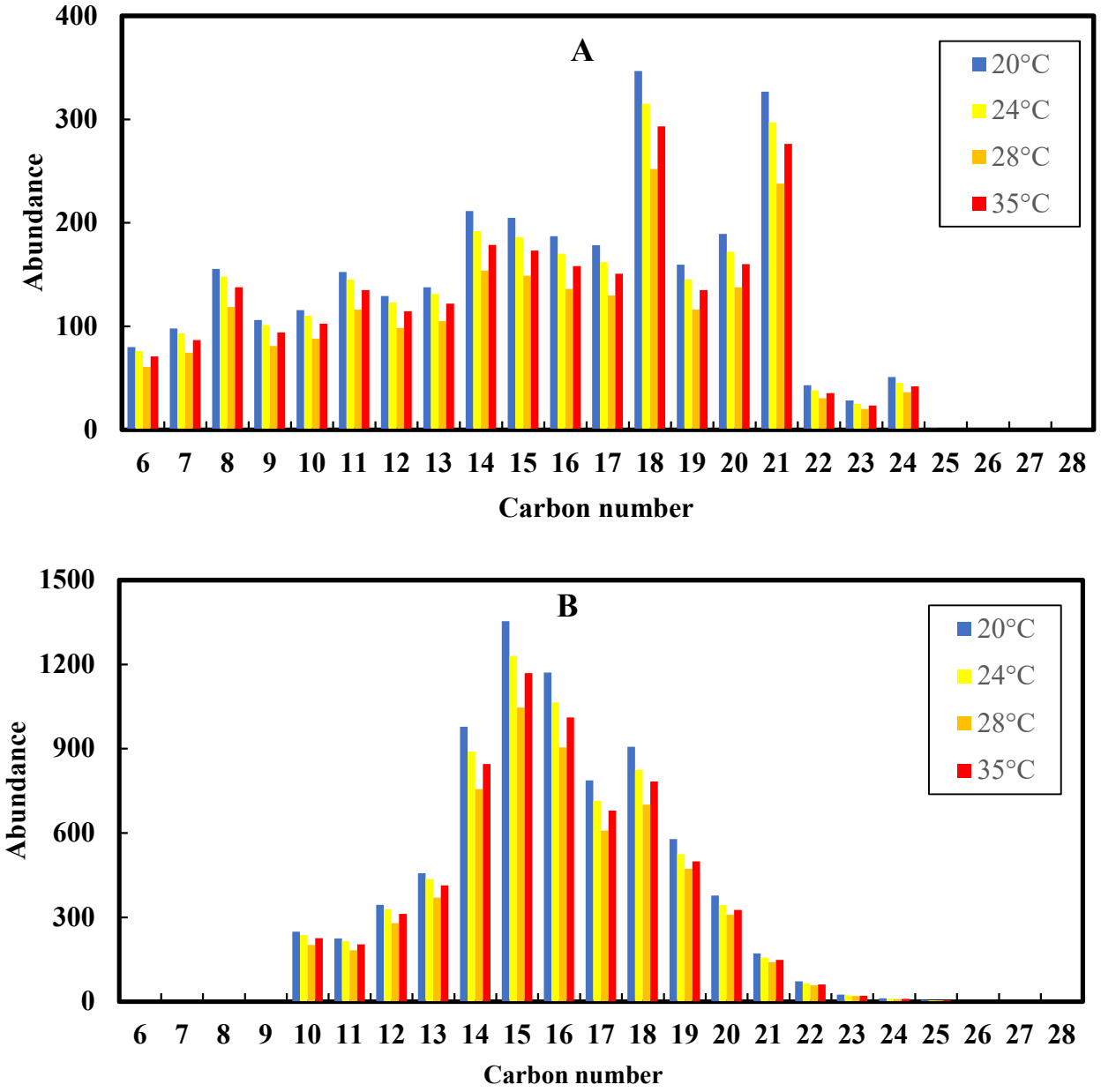


Figure 4.7 Distribution of naphthenic acids in batch cultures at different temperatures (initial concentration of 100 mg NA L^{-1} prepared in McKinney's medium) after 10 days biodegradation, Z= 0 (A), Z= -4 (B)

The obtained results of both panels A and B in Figure 4.7 confirm that although the minimum abundance of individual NAs were at 28°C, it was not distinctively lower than other temperatures. So that it can the concluded temperature variation did not effectively change the distribution of individual NAs. It can also be inferred that compared to NAs with higher carbon

numbers, the temperature increase had less effect on low molecular weight NAs residual abundance. In other words, these NAs have easily undergone biodegradation even at low or high temperatures, whereas heavier NAs favor optimal temperature of 28°C. To correlate fingerprint of individual NAs with Z number, comparison of panels A and B showed that at all temperatures, individual NAs without cyclicity were removed more than NAs with higher cyclicity.

4.2. Continuous biodegradation of commercial naphthenic acids in CPBBs

Continuous biodegradation experiments were carried out to evaluate the effectiveness of CPBBs to remove commercial NAs and to investigate the impact of biodegradation on the toxicity of the generated effluent. To assess the effect of operating condition, the bioreactors were run under varying NAs initial concentrations and loading rates (hydraulic residence time or flow rate). Overall, 40 different operating conditions were tested to study the effect of the initial NA concentration (50, 100, 150, and 200 mg NA L^{-1}) and volumetric loading rate (0.78-874.00 mg TOC L^{-1} h^{-1}). At each initial concentration, various loading rates were examined by changing the volumetric flow rate of the influent. At each flow rate, enough time was given to establish the steady state condition as indicated in section 3.3.4. All the experiments were conducted at room temperature. The performance of the bioreactor was assessed by determining the removal percentage, the removal rate of the total carbon, and the composition of individual NAs as a function of the loading rate. In addition, the impact of biodegradation on the toxicity of effluents generated under various conditions was evaluated.

4.2.1. Biodegradation results

The results obtained for biodegradation of commercial NAs in CPBB at different initial concentrations and loading rates are illustrated in

Figure **4.8** and Figure **4.9**. Detailed data for these figures is presented in Table C.1-Table C.4 in appendix C. In these figures, NAs residual concentration (effluent concentration), removal percentage, and removal rate are presented as a function of NAs loading rate all in terms of TOC concentration. As can be seen in

Figure **4.8**, the residual TOC concentration gradually increased as the loading rate was increased and then remained constant. This in turn, led to an initial decrease in NA removal percentage until it reached a minimum and after that, removal percentage did not change (Figure 4.9). On the other hand, the removal rate increased with the increase of loading rate. As expected, the residual concentration, removal percentage, and removal rate varied when different initial concentrations of NAs were applied in the influent. To be more specific, at similar loading rates, higher initial concentrations of NA in the influent led to higher NA residual concentrations.

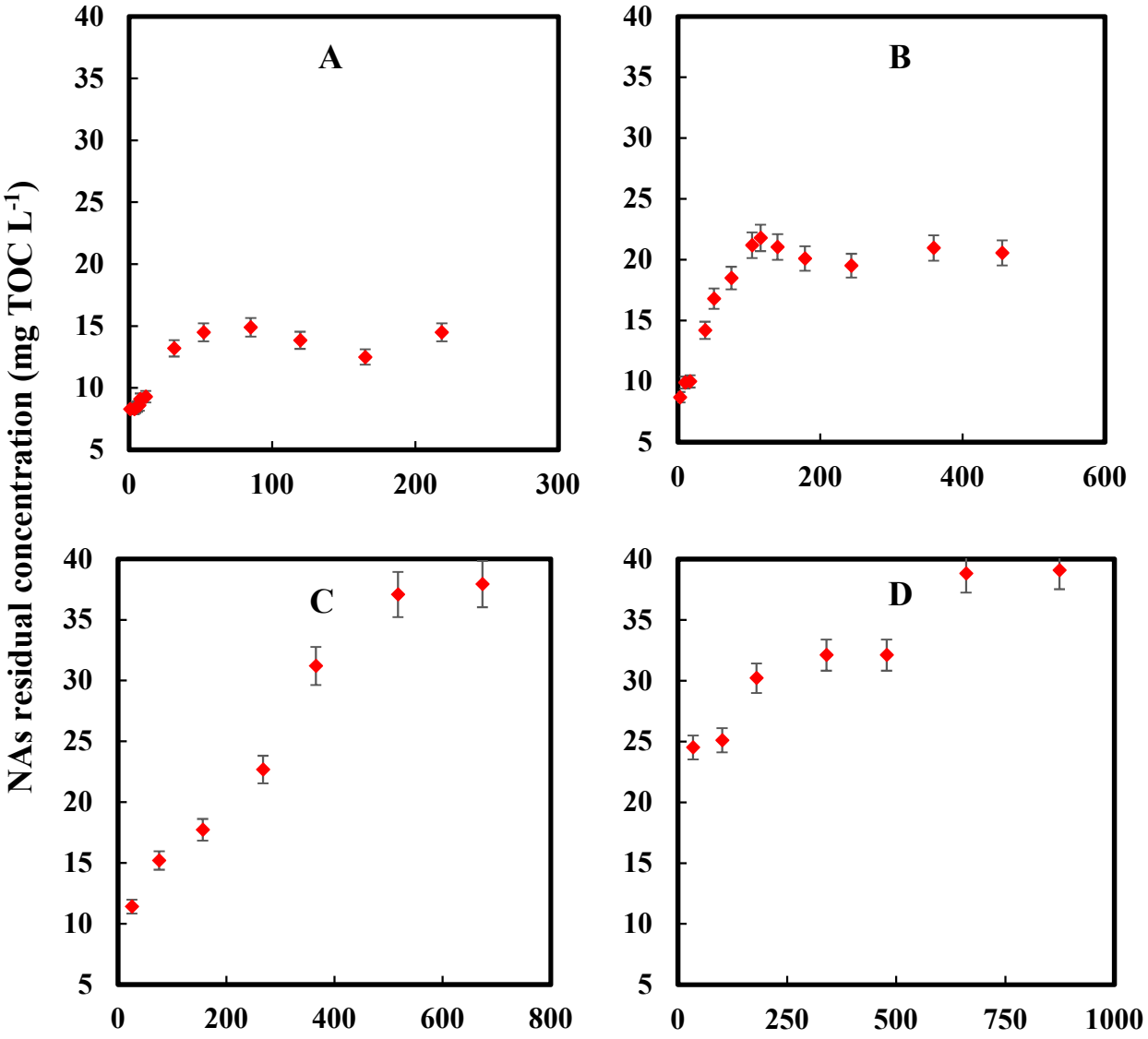


Figure 4.8 TOC variation of CPBB effluents with loading rate at various influent concentrations of commercial NAs: 50 mg NA L^{-1} (TOC=35.0±1.5 mg L^{-1}) (A), 100 mg NA L^{-1} (TOC=70.0±2 mg L^{-1}) (B), 150mg NA L^{-1} (TOC=104.0±3 mg L^{-1}) (C), 200mg NA L^{-1} (TOC=140.0±4 mg L^{-1}) (D)

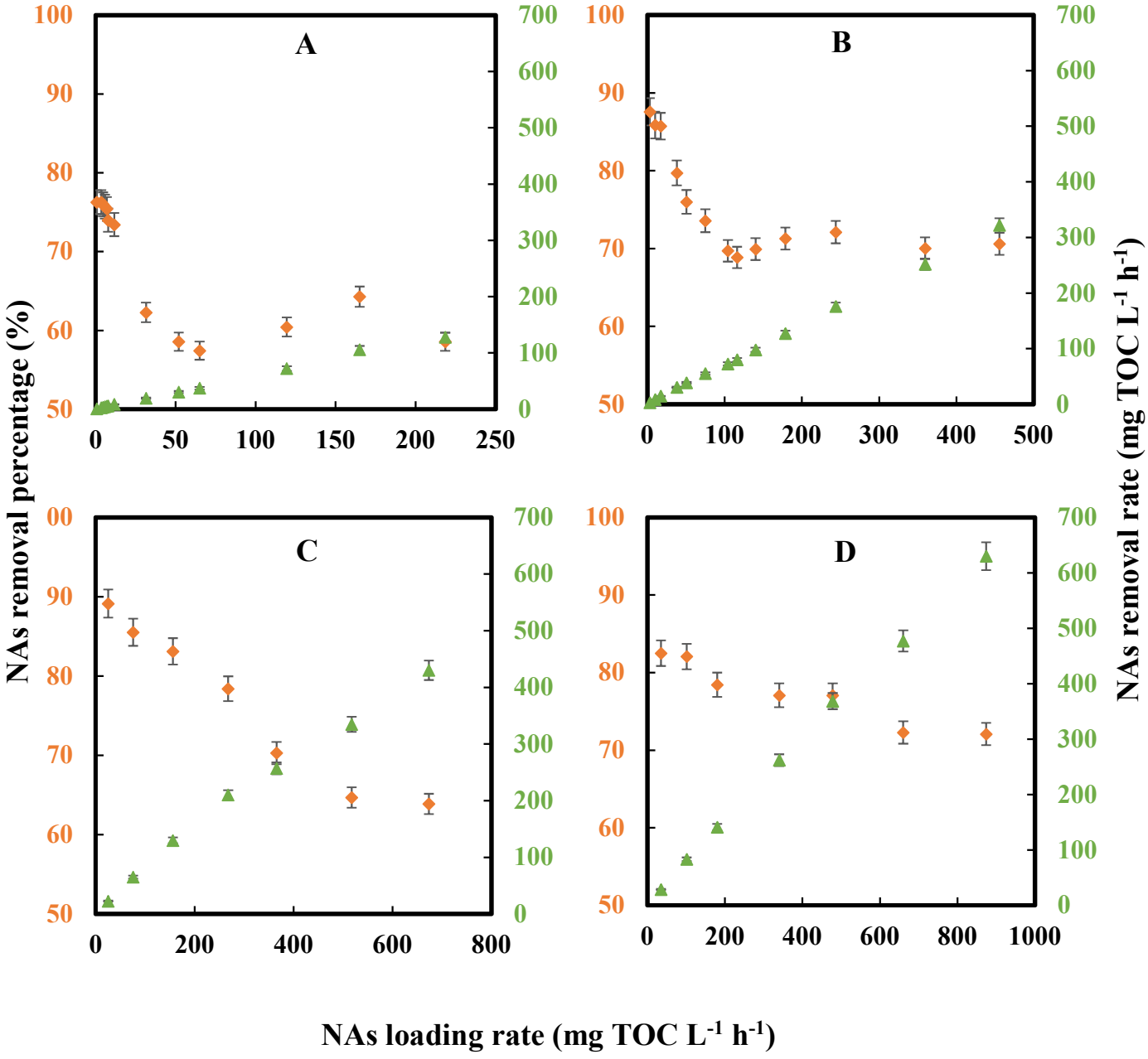


Figure 4.9 Removal percentage and removal rate of NAs as a function of NA loading rates for different influent NA concentrations: 50 mg NA L^{-1} (A), 100 mg NA L^{-1} (B), 150 mg NA L^{-1} (C), 200 mg NA L^{-1} (D). The error bars represent the standard deviations of data.

As shown in

Figure **4.8**A with the influent containing 50 mg NA L^{-1} (TOC concentration: 35.0 mg L^{-1}), at the lowest loading rate of 0.8 mg TOC L^{-1} h^{-1}, the residual NA concentration in the effluent was 8.3 mg TOC L^{-1}. At the high loading rate of 218.5 mg TOC L^{-1} h^{-1}, the residual NA concentration increased to 14.5 mg TOC L^{-1} which translates to 75% increase in residual concentration when compared to the lowest applied loading rate. These results also indicate that complete removal of NAs was not achieved.

With the NAs initial concentration of 50 mg NA L^{-1}, removal percentage of 77% was achieved at the lowest loading rate of 0.8 mg TOC L^{-1} h^{-1} with the corresponding removal rate of 0.6 mg TOC L^{-1} h^{-1} (residence time: 45 h). With further increase in the loading rate, the removal rate increased, while the removal percentage reached a minimum value and then remained relatively constant. To be more specific, an increase of loading rate to 85.0 mg TOC L^{-1} h^{-1} resulted in a decrease of removal percentage to around 57% which then fluctuated in the range 57 – 64% for loading rates up to 218.5 mg TOC L^{-1} h^{-1}. Removal rate had an increasing pattern with an increase of loading rate, with the maximum removal rate of 128.0 mg TOC L^{-1} h^{-1} obtained at the highest loading rate of 218.5 mg TOC L^{-1} h^{-1} (residence time: 0.16 h) (Figure 4.9A).

With an influent containing 100 mg NA L^{-1} (TOC=70.0±2 mg L^{-1}), at the lowest loading rate (3.0 mg TOC L^{-1} h^{-1}), the residual concentration in CPBB effluent was 8.7 mg TOC L^{-1}, while at the highest loading rate (455.6 mg TOC L^{-1} h^{-1}), a residual concentration of 20.6 mg TOC L^{-1} was obtained (

Figure **4.8**B). At this initial concentration of influent, a similar pattern was obtained for removal percentage and removal rate. In other words, with 100 mg NA L^{-1}in the influent, the increase of loading rate from 3.0 to 455.6 mg TOC L^{-1} h^{-1} increased the removal rate from 2.6 to 321.7 mg TOC L^{-1} h^{-1}. The initial NA removal percentage of 88% was achieved at the lowest loading rate (residence time: 23.33 h). The removal percentage gradually decreased to 68% at the loading rate of 116 mg TOC L^{-1} h^{-1} and then remained in the range of 68-72% for higher applied loading rates (Figure 4.9B).

In the next run the influent concentration was then increased to 150 mg NA L^{-1} (TOC=104.0±3 mg L^{-1}). With this concentration at the lowest loading rate (25.5 mg TOC L^{-1} h^{-1}), the residual concentration in CPBB was 11.4 mg TOC L^{-1} which increased to 37.9 mg TOC L^{-1} when the highest loading rate of 673.5 mg TOC L^{-1} h^{-1} was applied (

Figure **4.8**C). As illustrated in Figure 4.9C for the initial concentration of 150 mg NA L^{-1}, the loading rate was initially set at 25.50 mg TOC L^{-1} h^{-1} (residence time: 4.12 h) which resulted

in 90% NA removal percentage. The removal percentage then dropped by 63% when the highest loading rate of 430.2 mg TOC L^{-1} h^{-1} (residence time: 0.16 h) was applied. Likewise, the increasing pattern of removal rate from 22.7 to 430.2 mg TOC L^{-1} h^{-1} with increasing loading rate was achieved.

To assess the potential of removing high concentration NAs, higher initial concentration of 200 mg NA L^{-1} (TOC=140.0±4 mg L^{-1}) was applied and the same behavior was observed. Increasing the loading rate from 34.8 to 874.0 mg TOC L^{-1} h^{-1} led to a decrease in effluent concentration from 24.5 to 39.0 mg TOC L^{-1} (

Figure **4.8**D). For this high concentration, the removal percentage varied from 83% to 72% with changing loading rates from 34.8 to 874.0 mg TOC L^{-1} h^{-1} (residence time: 4.02 h-0.16 h). At the same time, an increasing variation of removal rate between 28.7 to 630.0 mg TOC L^{-1} h^{-1} was observed (Figure 4.9D).

These results, in general, showed that at each influent concentration an increase in loading rate led to a decrease in removal percentage which then stabilized at a certain level, whereas the removal rate increased. A possible explanation for this finding is that at lower loading rate the residence time increases. It means that NAs reside in the reactor for a longer time, which in turn lead to more bioconversion. For the increase in removal rate, the reason lies in the fact that the volumetric removal rate encompasses two factors; conversion and residence time. These two factors have an opposing effect. By increasing the loading rate, the conversion decreases which leads to a decrease in removal rate. The residence time also decreases; however, it causes an increase in removal rate. This combination leads to an overall increase in removal rate, as the effect of conversion is not as significant as the effect of residence time. In fact, the conversion at a higher loading rate reaches the lowest value and then remains constant.

In the preceding section, the impacts of NAs initial concentration and loading rate on biodegradation and associated removal rates were discussed. In order to assess the effect of biodegradation on the composition of NA mixture in terms of structure and molecular weight distribution, effluent samples obtained under different operating conditions were also analyzed by GC-MS and compared with that of influent. The selected total ion chromatograms (TIC) of NAs in the influent samples (untreated NA mixture, 200 mg NA L^{-1}) appeared as a hump of unresolved compounds (Figure 4.10A). This hump means that the original NA mixture is so complex that GC cannot separate all the components. Contrary to that, the total ion chromatograms of the CPBB effluent samples at different loading rates eluted with a noticeable

change (Figure 4.10B- H). As it can be seen, with higher residence time or lower loading rate (34.8-478.0 mg TOC L^{-1} h^{-1}) a marked change occurred which resulted in isolated peaks without a hump (Figure 4.10 D- H). On the other hand, at high loading rates (660.0-874.0 mg TOC L^{-1} h^{-1}), the change in TIC was different from those in lower loading rates (i.e. parts of the unresolved hump still existed). However, comparing with the influent TIC, it is clear that biodegradation not only removed the NAs but also impacted the distribution and/or composition of NAs (Figure 4.10 B-C).

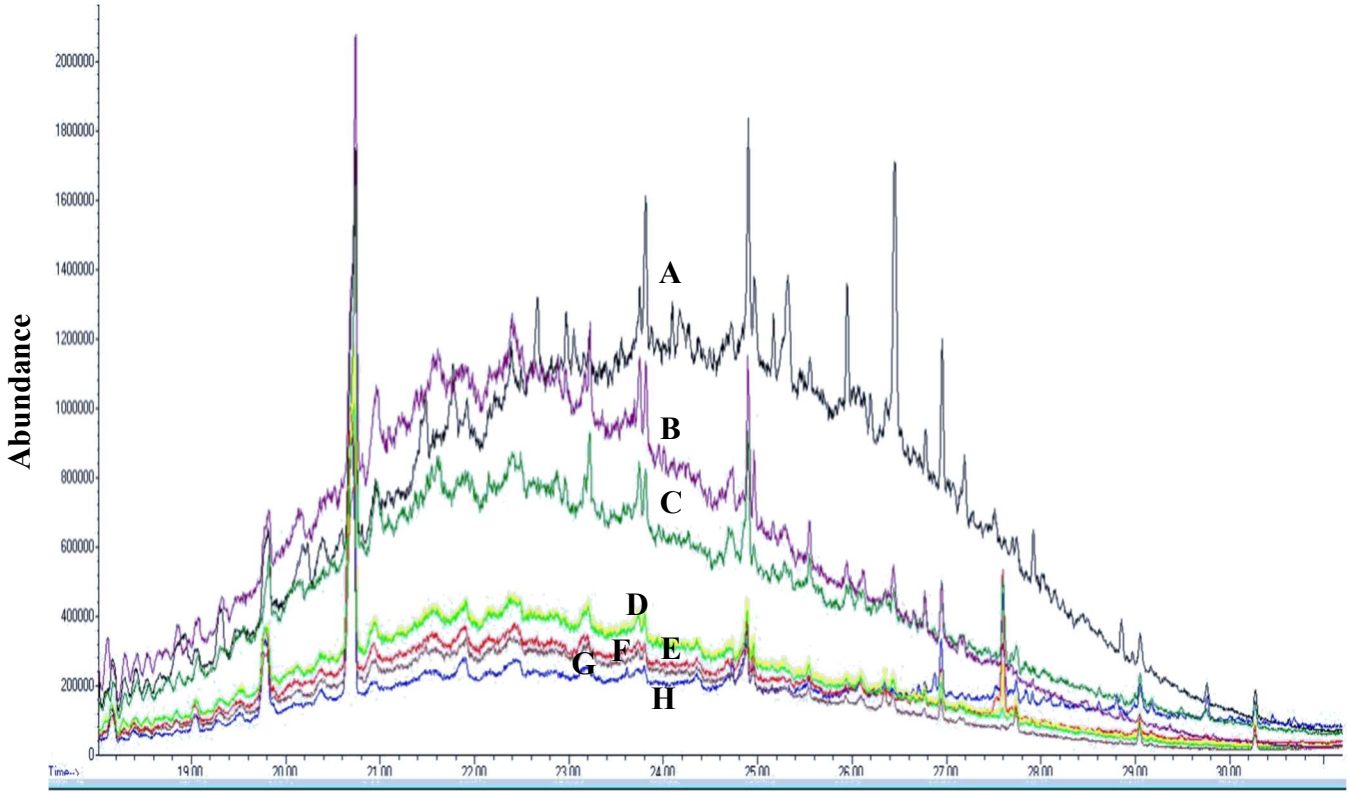


Figure 4.10 Total ion chromatograms of t-BDMS derivatized commercial NA mixture with influent containing 200 mg NA L^{-1} (140±4 mg TOC L^{-1}) (A), Effluent at loading rate of 874.0 mg TOC L^{-1} h^{-1} (B), effluent at loading rate=660.0 mg TOC L^{-1} h^{-1} (C), loading rate=478.0 mg TOC L^{-1} h^{-1} (D), loading rate=340.0 mg TOC L^{-1} h^{-1} (E), loading rate=180.0 mg TOC L^{-1} h^{-1} (F), loading rate=101.2 mg TOC L^{-1} h^{-1} (G), loading rate=34.8 mg TOC L^{-1} h^{-1} (H)

The similar TIC patterns were also obtained when different influent concentrations were applied (50,100, and 150 mg NA L^{-1}). These influents had unresolved humps which they disappeared to some extent after treatment in CPBBs. This removal extent was completely a function of loading rate where the lowest loading rates resulted in fewer residual peaks. Selected results of GC-MS analyses of samples obtained from the CPBB are presented as three-dimensional graphs. Influent molecular weight distribution, as well as CPBB effluents with the lowest, medium, and highest loading rate are plotted to be able to track changes of all individual NAs.

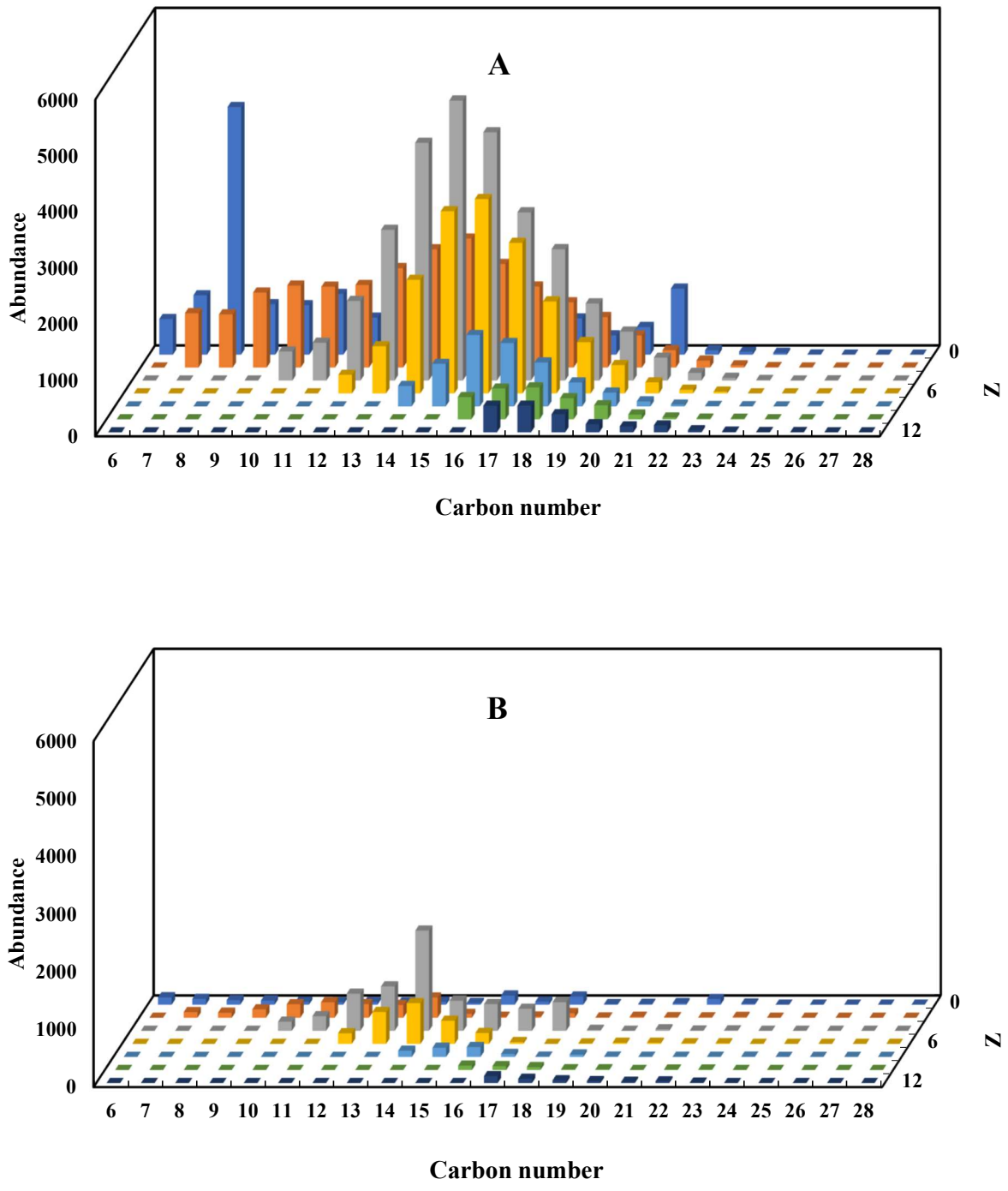
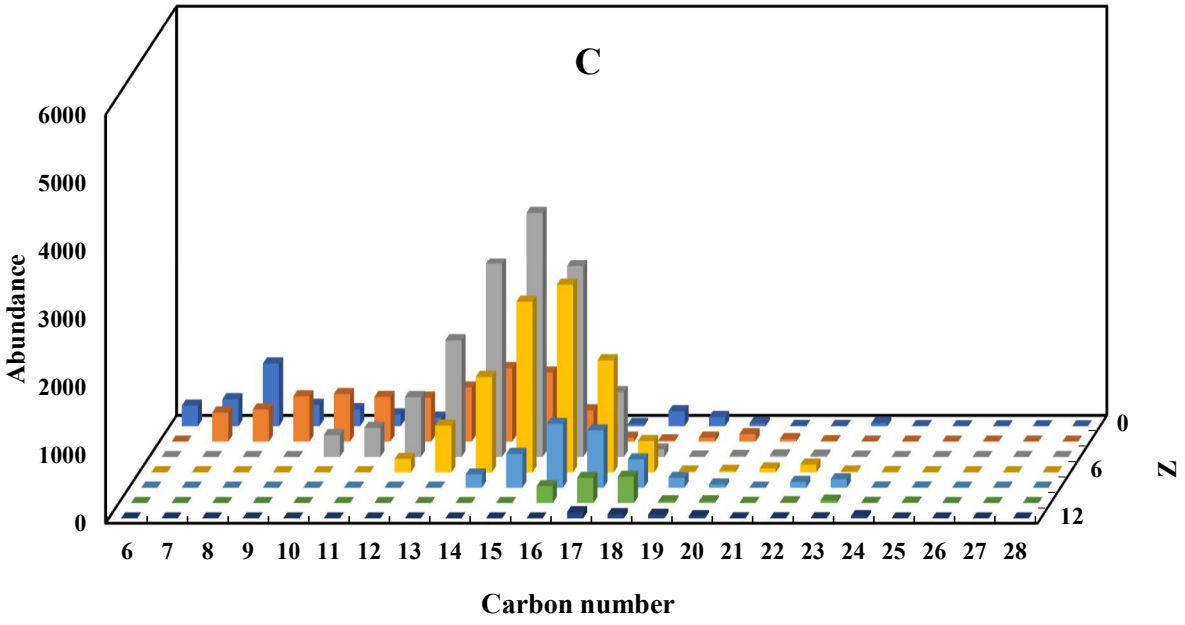
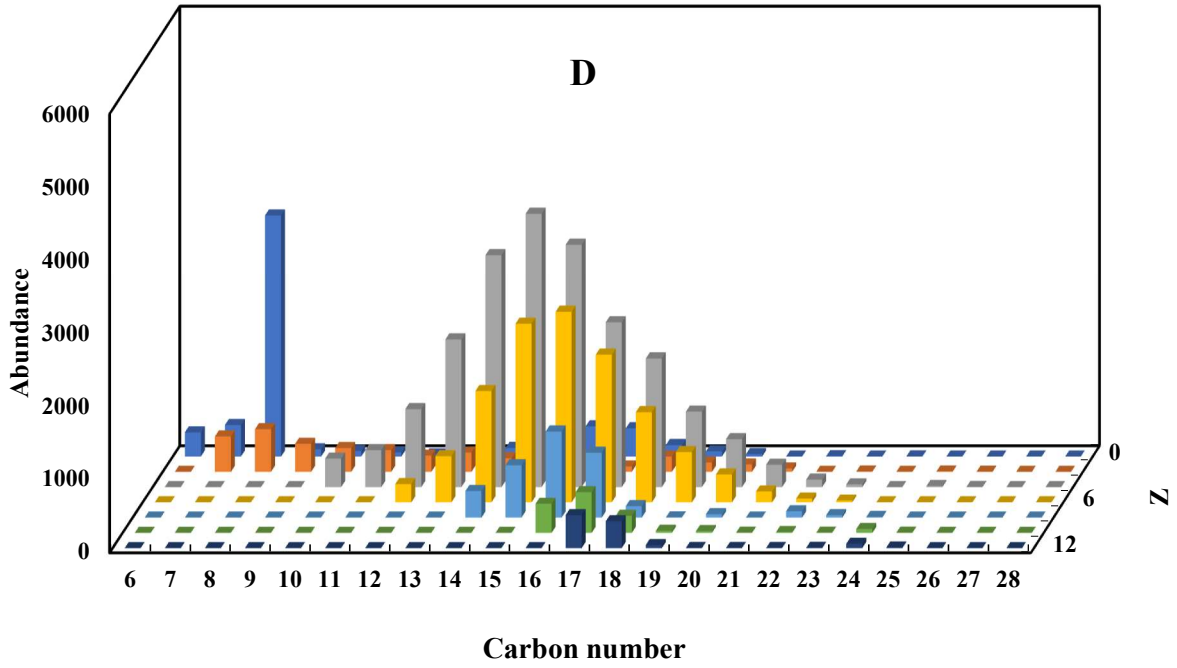


Figure 4.11 The distribution of naphthenic acids in the influent containing 50 mg NA L^{-1} and effluents obtained in the CPBBs. Influent (A), effluent at loading rate of 7.0 mg TOC L^{-1} h^{-1} (90 ml h^{-1}) (B) and effluent at loading rate 85.0 mg TOC L^{-1} h^{-1} (850 ml h^{-1}) (C) and effluent at loading rate 218.5 mg TOC L^{-1} h^{-1} (2185 ml h^{-1}) (D).

Figure 4.11 Continued





Comparing the distribution of NAs in the influent and effluent streams reveals that the concentration of most individual NA decreased as a result of biodegradation (Figure 4.11). Although it is hard to identify particular trends for the removal of NAs species, the distributions of carbon number and Z-family data of the samples before and after treatment indicate that in general low molecular weight NAs were preferentially degraded compared to high molecular weight NAs, and that the removal percentage of NAs was higher at the lower loading rates.

Due to the complex matrix of NAs mixture, the identification of the NA metabolites/ intermediates generated after treatment is very challenging and further work is needed to determine these molecules. Previous studies have noted the importance of chemical structure and molecular weight of NAs on the biodegradation rate (Herman *et al.*, 1994; Headley *et al.*, 2002; Biryukova *et al*., 2007; Huang *et al.*, 2012). For non-branched carboxylic acids, the addition of methyl groups hindered β-oxidation, and only mixed bacterial populations degraded recalcitrant NAs with methyl substitutions on the cycloalkane rings (Whitby, 2010). Differences in NA degradation rates for different geometric isomers have also been observed, whereby intramolecular hydrogen bonding occurring with the cis-isomers makes them less bioavailable and more difficult to metabolize (Huang *et al*., 2012; D'Souza *et al*., 2014).

The proposed approach by Clemente *et al.* (2004) assists in evaluating the effect of molecular weight on the extent of biodegradation. As discussed before in this method, the data in each matrix were divided into three groups. Group 1 contained the intensities for all of the naphthenic acids with carbon numbers of 5–13. Group 2 contained those with carbon numbers of 14–21, inclusive, and group 3 included those with carbon numbers of 22 and more. According to Figure 4.11 B-D and the associated data that is summarized in Table 4.3, at the lowest loading rate (7.0 mg TOC L^{-1} h^{-1}), 83.1% of C_5-C_{13} fraction and 81% of NAs in the range C_{14}-C_{21} were removed, while for the C_{22}^{+} NAs the removal was 74.7%. The similar pattern was observed when higher loading rates were applied. At the loading rate of 85.0 mg TOC L^{-1} h^{-1} removal percentage of lighter NAs (C_5-C_{13}) was 57.1% and a lower percentage of 43.6% was observed for heavier NAs (C_{22}^{+}). Likewise, at the loading rate of 218.5 mg TOC L^{-1} h^{-1}, 54.7%, and 44.2% removal percentage were achieved for low and high molecular weight NAs. These findings show that NAs with lower molecular weight were more amenable to biodegradation when compared to those with higher molecular weight.

To explain different patterns of biodegradation of various surrogate NAs in a mixture, D'Souza *et al*. (2014) suggested two different mechanisms. First, direct metabolism of NAs by the microbial culture, which appears to be the dominant mechanism for those NAs amenable to biodegradation and to a lesser extent for recalcitrant NAs. Second, degradation of recalcitrant NAs by the enzymes which are produced during the metabolism of NAs amenable to biodegradation. Thus, it could be suggested that with a mixture of NAs microbial population metabolized all compounds simultaneously, but some NAs were used as the preferred substrates, resulting in higher removals for them.

Table 4.3 NAs percent removal as a function of NA loading rate with an influent containing 50 mg NA L^{-1} (35±1.5 mg TOC L^{-1})

		Removal Percentage (%)		
		Loading rate (mg TOC L^{-1} h^{-1})		
Fraction	**N**	**7.0**	**85.0**	**218.5**
Light	6-13	83.1	57.1	54.7
Medium	14-21	81.0	55.9	42.9
Heavy	22+	74.7	43.6	44.2

The extent of biodegradation also depended on Z number, which represents hydrogen deficiency number as an indicator of cyclicity or unsaturated bond(s). According to Figure 4.11 and Table 4.4 that shows the dependency of NAs removal on Z number, at the lowest loading rate of 7.0 mg TOC L^{-1} h^{-1}, the removal percentages were 92.8, 90.7, 79.8, 86.6, 89.2, 89.6, and 80.1% for Z values of 0, -2, -4, -6, -8, -10, -12, respectively. This high removal efficiency of NAs in all Z values could be attributed to the ability of developed biofilm and characteristics of CPBB to degrade NAs. These percentages changed to 79.0, 64.4, 49.5, 38.2, 35.7, 51.4, and 77.5% for Z values of 0 to -12 at loading rate of 85.0 mg TOC L^{-1} h^{-1}. A further decrease to 63.7, 62.6, 25, 25, 32.3, 43.1, 45.1% for Z values of 0 to -12 occurred for the highest loading rate of 218.5 mg TOC L^{-1} h^{-1}. These results indicate that no obvious pattern in terms of Z number effect on NAs removal can be established which is in agreement with the results of previous studies (Martin *et al.*, 2010; Islam *et al.*, 2014). Analysis of commercial and OSPW NA by GC × GC–MS by others has shown that the ions detected by high-resolution mass spectrometry alone

represent hundreds of different structural isomers which make it even harder to find a proper mechanism and reaction pathway (Wilde & Rowland, 2018). However, in general, NAs with fewer rings are more susceptible to biodegradation because the presence or increase of cyclicity in the structure of NAs can increase the hydrophobicity of the compound (D'Souza *et al*., 2014)). This also accords with Del Rio *et al.*, (2006) observation that the bicyclic (Z= -4) surrogates are less amenable to biodegradation than the monocyclic (Z= -2) surrogates.

Table 4.4 NAs percent removal of CPBB effluents based on Z number at an initial concentration of 50 mg NA L^{-1} (35±1.5 mg TOC L^{-1})

	Removal Percentage (%)		
	Loading rate (mg TOC L^{-1} h^{-1})		
Z	**7.0**	**85.0**	**218.5**
0	92.8	79.0	63.7
-2	90.7	64.4	62.6
-4	79.8	49.5	25.0
-6	86.6	38.2	25.0
-8	89.2	35.7	32.3
-10	89.6	51.4	43.1
-12	80.1	77.5	45.1

Surprisingly, the removal of more complex NAs with the highest number of rings (Z= -12) appeared to be higher than less cyclic NAs (Z= -4, -6, -8), specifically for higher loading rates. A similar pattern has been reported where integrated adsorption and ozonation was utilized to detoxify OSPW (Gamal El-Din *et al*., 2011; Zubot *et al*., 2012). The observed pattern in case of the adsorption process can be related to higher hydrophobicity and less solubility of NAs with a higher number of the carbons and rings. The difference in solubility results in a higher affinity of larger molecules to adsorb onto the adsorbent, which could improve the adsorption of NAs. So, by considering the same phenomenon, the adsorption of larger NAs on the surface of biofilm

can increase the removal percentage. This observation for Z= -12 also might be due to the presence of NA-like compounds with multi- carboxylic groups in their structures which degrade faster compared to less cyclic NAs. Additionally, lower removal of NAs with Z= -4, -6, and -8 may indicate a conversion via biodegradation of NAs with Z = -10 and -12 to NAs with Z= -4, -6, and -8 (Islam *et al.*, 2014).

The GC-MS analysis was also utilized for CPBBs effluents with higher NAs influent concentration, and selected results for the highest NAs initial concentration (200 mg NA L^{-1}) are presented in Figure 4.12. Comparing with the GC-MS results of CPBBs effluents with an influent initial concentration of 50 mg NA L^{-1} (Figure 4.11 and Figure **4.12**) shows that the biofilm is still capable of removing higher concentration. However, the removal is accompanied by the higher residual NAs in CPBBs effluent and lower removal percentages, especially at high loading rates. It should be noted that the removal levels reported in this study do not necessarily indicate mineralization. Transformed NAs may have lower ionization efficiencies or elute outside of the range used for quantification.

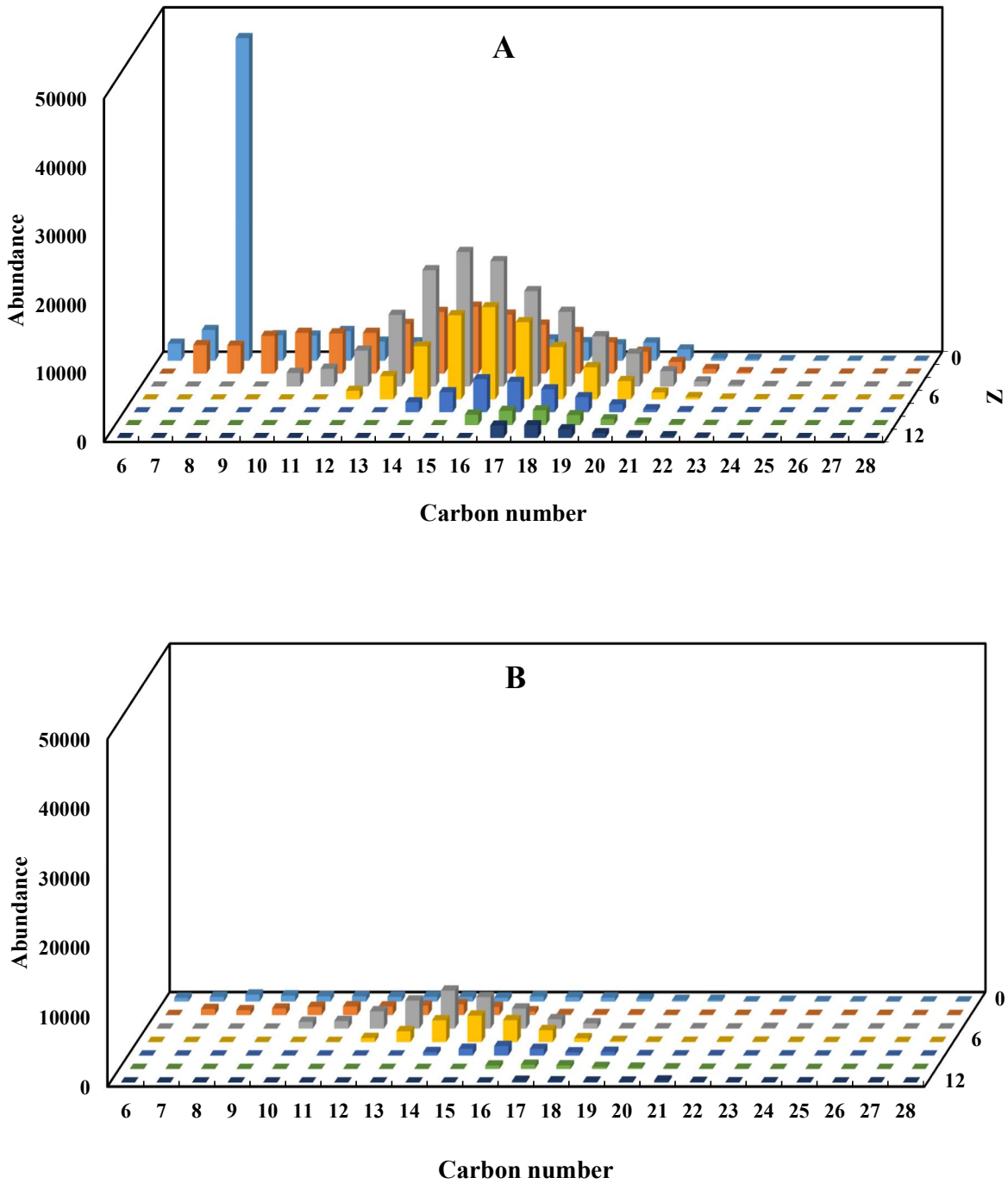
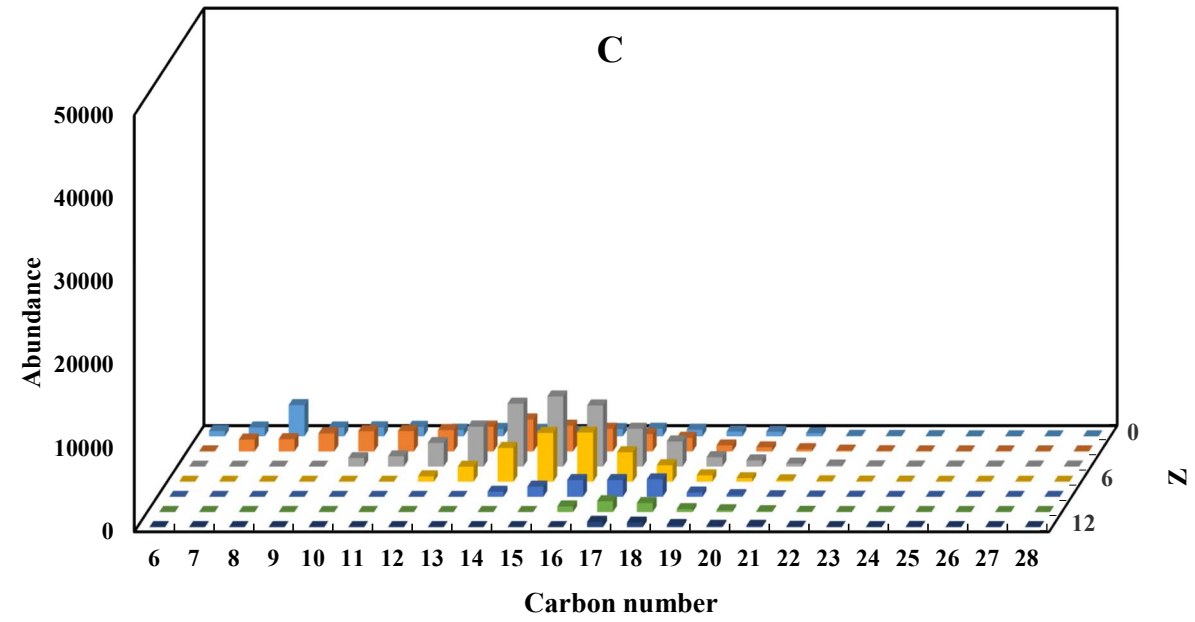
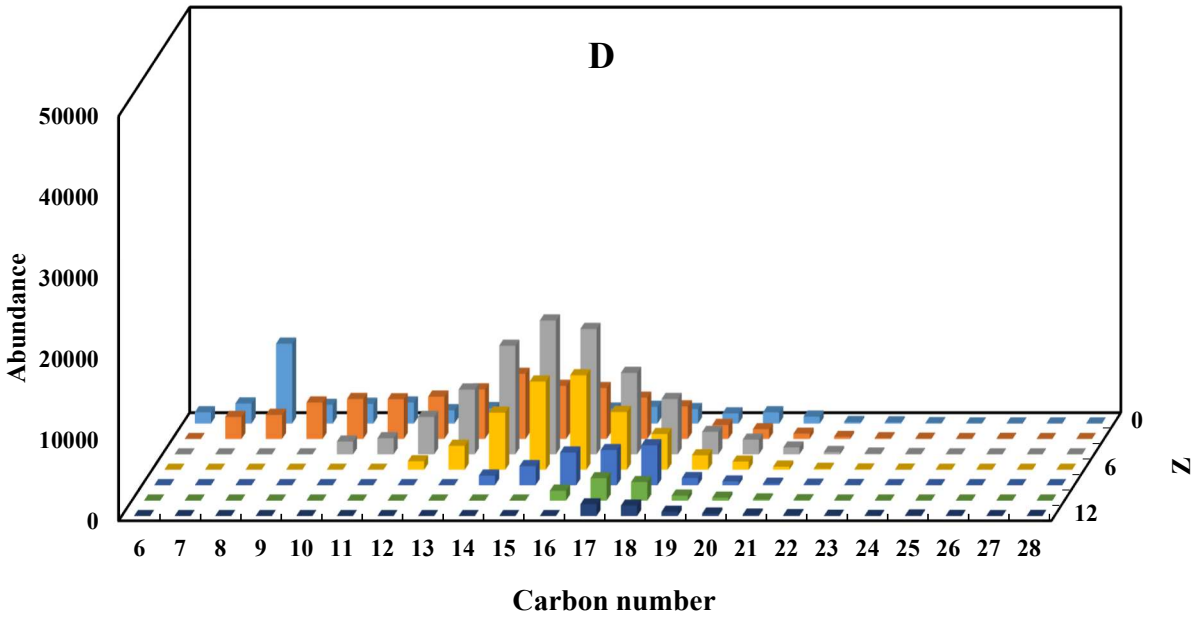


Figure 4.12 The distribution of naphthenic acids in the influent containing 200 mg NA L^{-1} and effluents obtained in the CPBBs. Influent (A), effluent at loading rate of 34.8 mg TOC L^{-1} h^{-1} (87 ml h^{-1}) (B) and effluent at loading rate 340.0 mg TOC L^{-1} h^{-1} (850 ml h^{-1}) (C) and effluent at loading rate 874.0 mg TOC L^{-1} h^{-1} (2185 ml h^{-1}) (D).

Figure 4.12 Continued





Further investigation of results showed that at the loading rate of 34.8 mg TOC L^{-1} h^{-1}, 81.9% of low molecular weight (C_5-C_{13}) NAs and 80.5% of NAs in range of C_{14}-C_{21} carbon number were removed. Increasing loading rate to 340.0 mg TOC L^{-1} h^{-1} led to removal percentage of 71.3% and 65.5% for these groups. Applying the highest loading rate of 874.0 mg TOC L^{-1} h^{-1} caused 63.4% and 28.7% reduction in the concentration of the original substrate NAs. This data which is summarized in Table 4.5 agrees with other findings which molecular weight had a direct effect on biodegradation (Han *et al.*, 2008; Toor *et al.*, 2013; D'Souza *et al.*, 2014).

Table 4.5 NAs percent removal of CPBB effluents based on carbon number (n) at an initial concentration of 200 mg NA L^{-1} (140±4 mg TOC L^{-1})

		Removal percentage (%)		
		Loading rate (mg TOC L^{-1} h^{-1})		
Fraction	**n**	**34.8**	**340.0**	**874.0**
Light	6-13	81.9	71.3	63.4
Medium	14-21	80.5	65.5	28.7
Heavy	22+	78.1	69.9	57.3

Likewise, finding a pattern for the cyclicity effect on the performance of CPBBs to remove NAs is difficult. The data of Table 4.6 indicates that CPBBs had the highest efficiency for acyclic NAs and having more cyclicity in NAs structure led to a complex pattern of biological treatment. As a result, given the complexity of the NA matrix in commercial NAs, it is inferred that the biodegradation of NAs with various molecular structures may be influenced differently by variation of loading rate. For instance, the removal of classical NAs with shorter carbon chains and low cyclicity was more sensitive to a loading rate change as opposed to their counterparts with longer carbon chains and more rings.

It should also be noted that a similar pattern was seen for the other influent concentrations. Similarly, for 100 and 150 mg NA L^{-1} influent concentration, NAs with 5-13

carbons and the least cyclicity had the highest removal percentage which increasing loading rate had a negative effect on it.

Table 4.6 NAs percent removal of CPBB effluents based on Z number at an initial concentration of 200 mg NA L^{-1} (140±4 mg TOC L^{-1})

	Removal percentage (%)		
	Loading rate (mg TOC L^{-1} h^{-1})		
Z	**34.8**	**340.0**	**874.0**
0	89.1	83.2	61.1
-2	87.2	65.8	27.1
-4	79.2	62.4	26.7
-6	77.5	61.5	26.1
-8	76.5	57.9	12.1
-10	74.1	56.8	13.2
-12	84.4	71.3	38.4

Results obtained in the current work were consistent with the past research that was conducted in the past for the surrogate NAs. For the biodegradation of trans-4-methyl-cyclohexane carboxylic acid (trans-4MCHCA), the maximum reaction rates of 9.6 mg TOC L^{-1} h^{-1} at a residence time of 40 h and 917.0 mg TOC L^{-1} h^{-1} at a residence time of 2.6 h were observed in the continuously stirred tank and immobilized cell bioreactors, respectively (Paslawski *et al*., 2009). The maximum trans-4MCHCA biodegradation rate reported by Huang *et al.* in a continuous CPBB was 209.0 mg TOC L^{-1} h^{-1} at a residence time of 0.15 h (Huang *et al*., 2012). The biodegradation rates of cis- and trans-4-MCHAA were much lower than trans-4MCHCA, with the maximum biodegradation rates determined for the two isomers being 4.2 and 7.8 mg TOC L^{-1} h^{-1}, respectively (residence time: 3.3 h).

4.2.2. Toxicity evaluation of CPBBs influents and effluents

As discussed in the chapter 2, naphthenic acids demonstrate toxicity toward a variety of species (Kannel & Gan., 2012). Developed biofilm in the CPBB has shown its ability to remove commercial NAs (fully or partially depending on the applied loading rate). However, it is

important that the treated effluents be examined from a toxicological point. The outcome of this evaluation is of great importance as it verifies to what extent the CPBB was able to treat the influents. Influent samples containing various levels of commercial NAs (50, 100, 150, and 200 mg NA L^{-1}) were assessed to evaluate their toxicity toward *A. Salina*. Moreover, the toxicity of CPBB treated effluents obtained at different loading rates with various levels of NAs in the feed were also evaluated to assess the detoxifying ability of the CPBBs and developed biofilms.

Representative results of the toxicity evaluation are summarized in Figure **4.13**. Results of control experiment indicated that *A. salina* (shrimp larvae) was not sensitive to the modified McKinney's medium, indicating that the mineral nutrients at the level present in this medium did not impose a toxic effect. Specifically, in almost all cases, 100% shrimp larvae remained alive within 4 h exposure.

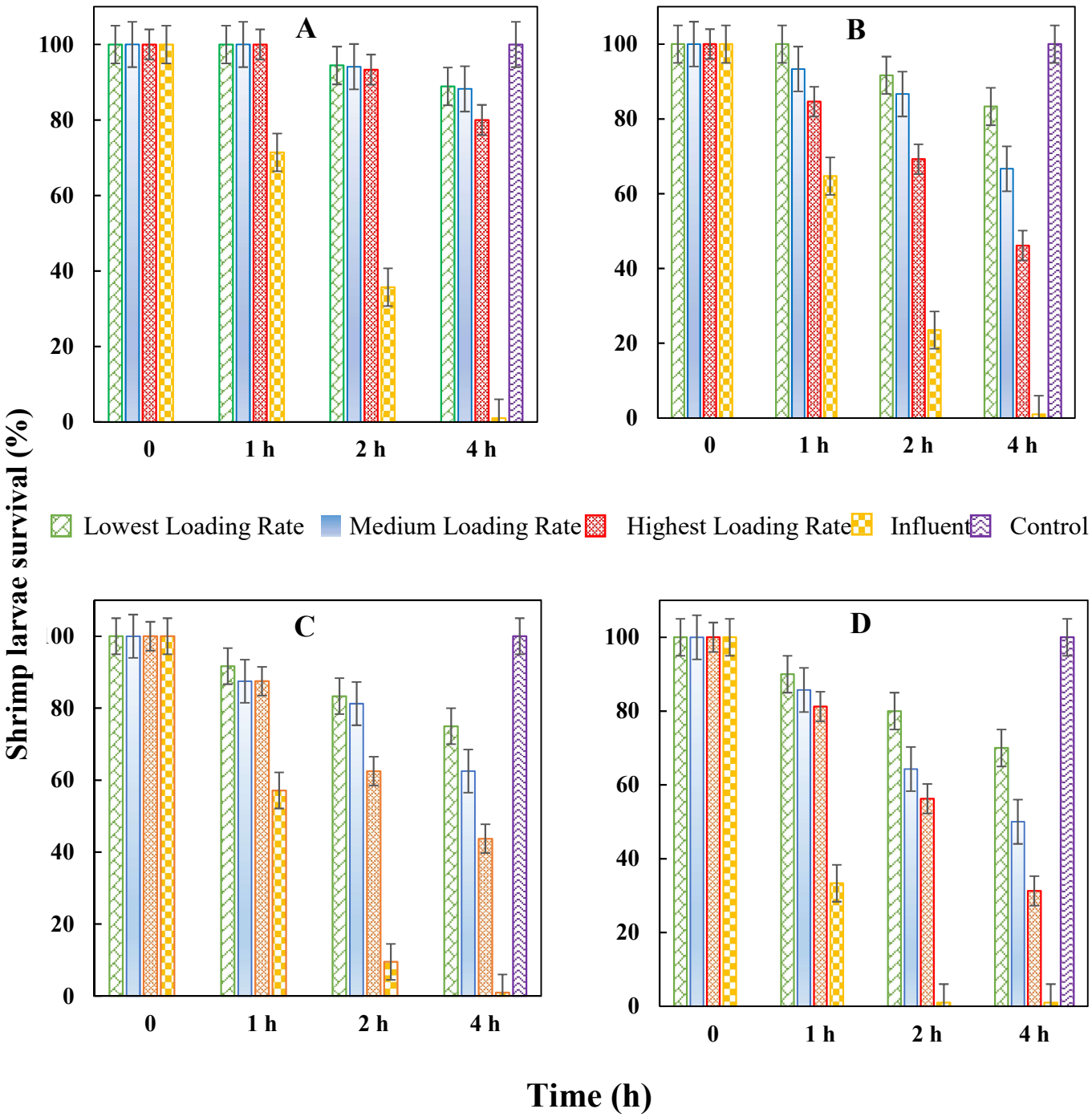


Figure 4.13 Results of A. salina toxicity evaluation on the CPBB untreated influents, treated effluents at different loading rates, and control samples. Results are provided for various initial concentrations of commercial NAs: 50 mg NA L^{-1} (A), 100 mg NA L^{-1} (B), 150 mg NA L^{-1} (C), 200 mg NA L^{-1} (D).

With influent containing 50, 100, 150, and 200 mg NA L^{-1} commercial NAs and after 2 h exposure, the survival levels of shrimp larvae were 35.7, 23.5, 9.5 and 0%, respectively and after 4 h, all shrimp larvae died for even lower level concentrations. These numbers indicated that commercial NAs were moderately toxic at a low concentration of 50 mg NA L^{-1} and increasing the concentration noticeably increased toxicity levels.

In

Figure **4.13**, for effluents, shrimp larvae survival results are shown at specific loading rates. At each influent concentration, lowest loading rate is the loading rate in which the removal percentage is the highest, while the removal rate is low. Therefore, highest loading rate corresponds to the moderate removal percentage and the highest removal rate. For the effluent generated during the biodegradation of 50 mg NA L^{-1} commercial NAs with the lowest loading rate of 7.0 mg TOC L^{-1} h^{-1} (NAs removal percentage: 75.4%), the survival of shrimp larvae after 4 h was 88.9%. Increasing the loading rate to 218.5 mg TOC L^{-1} h^{-1} (NAs removal percentage: 58.6%) led to a slight decrease of shrimp larvae survival (80%). In the case of an influent with 100 mg NA L^{-1} commercial NAs, at the lowest loading rate of 17.0 mg TOC L^{-1} h^{-1} (NAs removal percentage: 85.7%), after 4 h 83.33% of shrimp larvae survived. It was accompanied by the survival of 46% at the loading rate of 455.6 mg TOC L^{-1} h^{-1} (NAs removal percentage: 70.6%). Further increase of influent concentration to 150 mg NA L^{-1} led to the death of more shrimp larvae. At the lowest loading rate of 25.5 mg TOC L^{-1} h^{-1} (NAs removal percentage: 89.1%), after 4 h 75% of shrimp larvae stayed live. This survival dropped to 44% for the highest loading rate of 673.5 mg TOC L^{-1} h^{-1} (NAs removal percentage: 63.9%). Finally, with 200 mg NA L^{-1} commercial NAs, 70 % of shrimp larvae could survive at the lowest loading rate of 34.8 mg TOC L^{-1} h^{-1} (NAs removal percentage: 82.5%) after 4 h and it is then reduced to 31% at the maximum loading rate of 874.0 mg TOC L^{-1} h^{-1} (NAs removal percentage: 72.1%).

The *A. salina* test revealed that the survival rate was significantly higher in the treated effluents as compared with the corresponding untreated influent. So, the results of this test indicate that the treatment of influents in the CPBBs led to a marked decrease in toxicity, as the survival percentage of shrimp in the treated effluents were substantially higher when compared with the corresponding results for the untreated influent. It can also be concluded that the effluent toxicity reduction was proportional to the influent NAs concentration. Moreover, increasing the loading rate led to higher toxicity as it ended up with higher NAs residual. All in

all, lower influent commercial NAs concentration or lower loading rate resulted in higher reclamation.

Microtox toxicity tests were also conducted using the untreated influents (100 and 200 mg NA L^{-1}) and treated effluents at the lowest and highest loading rates, corresponding to highest removal percentage and removal rates, respectively. The Microtox results are presented in Table 4.7 in terms of IC_{50} (v/v %). This is the concentration of a toxic environment causing 50% inhibition of a specific biological or biochemical function. The high toxicity of the influent at a concentration of 100 mg NA L^{-1} with 5 min IC_{50} as 7.6% is reduced to a final 5 min IC_{50} of 78.1% after treatment at the lowest loading rate of 17.0 mg TOC L^{-1} h^{-1} and 41.7% for the highest loading rate of 455.6 mg TOC L^{-1} h^{-1}. In the case of 200 mg NA L^{-1} influent concentration, 5 min IC_{50} as 4.0% increased to 68.5% for lowest loading rate of 34.8 mg TOC L^{-1} h^{-1} and 36.5% for the highest loading rate of 874.0 mg TOC L^{-1} h^{-1} which was an indicator of a marked decline in toxicity.

Table 4.7 Microtox toxicity results for CPBB influents and treated effluents

Initial Concentration (mg NA L^{-1})	**IC_{50} 5min (%)**		
	Influent	**Effluent (lowest loading rate)**	**Effluent (highest loading rate)**
100	7.6	78.1	41.7
200	4.0	68.5	36.5

The remaining toxicity might be due to the residual NAs, which were not biodegraded. As mentioned before, NAs with higher molecular weight and more extensive ring formation were more biopersistent and more difficult to be removed. These remaining biopersistant NAs, which are hydrophobic compounds, usually more easily permeate the lipid bilayer, alter membrane function, and ultimately cause cell death. So, this observed reduction in toxicity even for highly concentrated samples in high loading rates can be attributed to the higher removal of lower molecular weight compounds in a NA mixture correlate with greater toxic response in various organisms (Brown & Ulrich ., 2015; Clemente *et al*., 2004; Lo *et al*., 2006). They suggested that the toxic potency of NAs is structure-dependent, which is affecting NAs

surfactant characteristics. As surfactants, they have lipophilic heads which can easily penetrate the cell wall of a biological membrane and disrupt its function (Kannel & Gan., 2012; Quagraine *et al.*, 2005).

The comparison of these two toxicity analyses showed that the results of brine shrimp method were confirmed by Microtox. So, the brine shrimp method can be used as a rapid and inexpensive test to analyze the change in toxicity.

4.2.3. Microbial community analysis

A sample of the developed biofilm in CPBBs, collected at the end of runs, was sent to an external laboratory to determine the composition of the developed microbial community as described in section 3.4.4. This analysis was conducted to the genus level, and results are presented in Figure 4.14.

As seen in Figure 4.14 (panel A) at the phylum level, most of the microbial community comprised *Proteobacteria* (90.93%). *Actinobacteria* (6.91%) and *Bacteriodetes* (1.00%) were also detected in the biofilm. Most *Proteobacteria* are mesophilic, motile by means of polar flagella, and free-living (non-parasitic). The high abundances of *Proteobacteria* may have resulted from the resistance of this phylum to the toxicity of NAs and the broad degradation ability of this phylum.

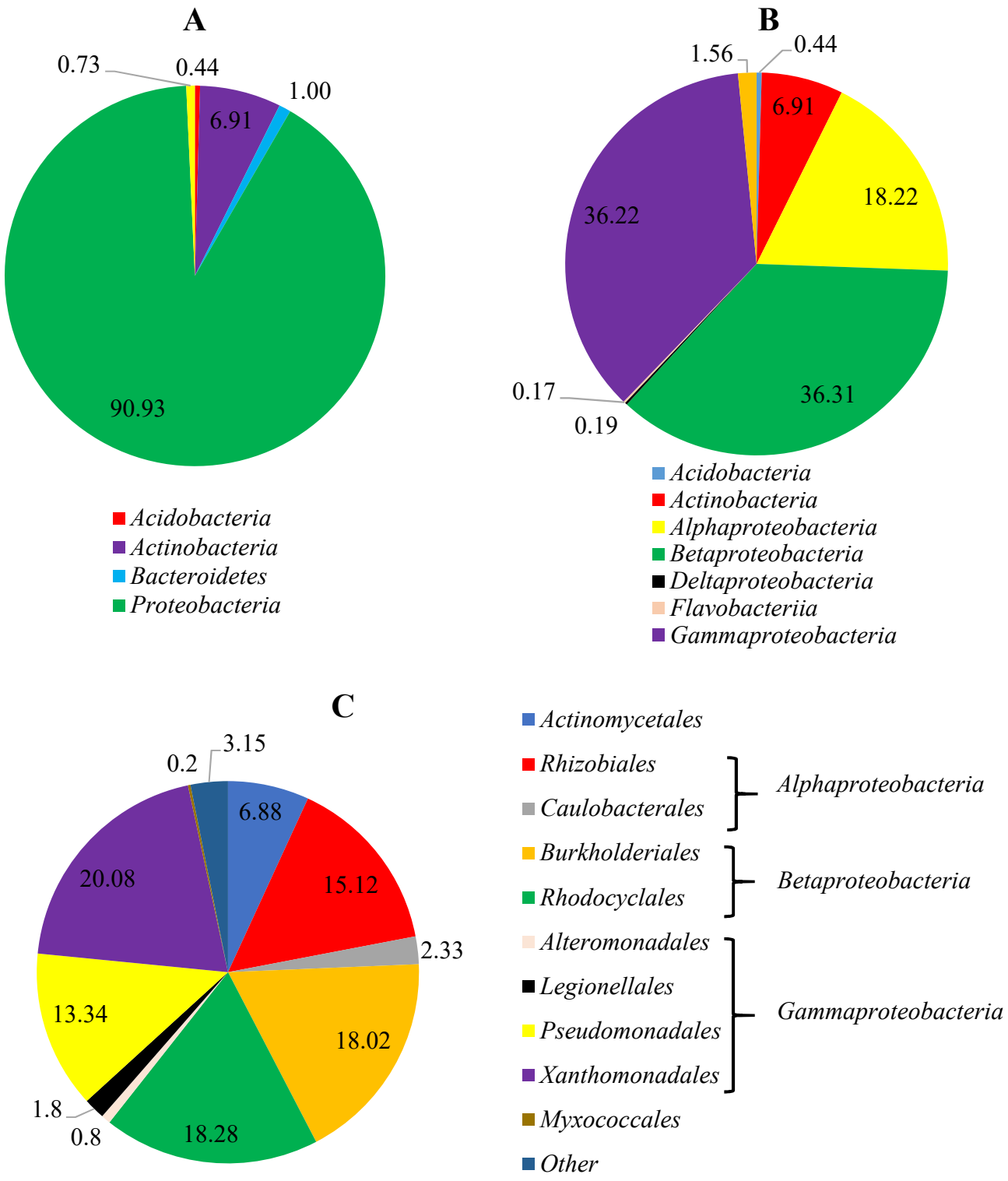


Figure 4.14 Microbial community profiles of the biofilm developed in the CPBB at the A) phylum level, B) class level, C) order level

In a previous study by Yergeau *et al*. (2012), the relative abundances of *Proteobacteria* were positively correlated with the concentration of naphthenic acids. Thus, because of the high concentration of NAs in CPBB (up to 200 mg NA L^{-1}), it is not surprising to have high proportions of this phylum. Moreover, several studies have reported that *Proteobacteria* were the dominant microorganisms in oil sand tailings ponds and in the Athabasca River and its tributaries which are able to remove recalcitrant NAs (Whitby, 2010; Yu *et al.*, 2018; Zhang *et al.*, 2015). *Actinobacteria* were also found in the developed biofilm community. They are mainly aerobes which are widespread in soil and play an important role in the biodegradation and recycling of organic matter (Trujillo, 2016).

Labrada & Nemati, (2018) found different microbial communities during the biodegradation of surrogate NAs in microbial fuel cells. In their work, in the biofilm developed with trans-4MCHCA, the most abundant phyla were *Proteobacteria* (48.60%) and *Bacteroidetes* (44.90%), while with octanoic acid in addition to *Proteobacteria* and *Bacteroidetes* that accounted for 57.41 and 17.48% of the community, *Actinobacteria* was also present at a substantial level of 17.37%. Comparing the current study with Labrada & Nemati, (2018) , which the same culture source has been used in both, it can be concluded that the complexity of NAs mixture affects the microbial community composition. It means that the response of the initial microbial community under various conditions can be changed significantly.

The class level examination of the microbial community in Figure 4.14 (panel B) showed that *Alphaproteobacteria* (18.22%), *Betaproteobacteria* (36.31%), and *Gammaproteobacteria* (36.31%) existed in the biofilm. Further investigation at the taxonomic order level revealed more information about the diversity of bacteria in the CPBB biofilm. Based on Figure 4.14, *Betaproteobacteria* are the dominant class of bacteria. Yergeau *et al*. (2012) observed that this class was the most abundant in or near tailings ponds. They also found that *Betaproteobacteria* are strong bitumen degraders in the oil sands tailing ponds. *Burkholderiales* and *Rhodocyclales* are the major orders of *Betaproteobacteria* (Figure 4.14 (panel C)). The relevance of the *Burkholderiales* order in degradation of a vast array of aromatic compounds, including several priority pollutants, has been widely reported (Pérez-Pantoja *et al*., 2012). *Rhodocyclales* are widespread and abundant in wastewater treatment systems and have diverse physiological capabilities that are beneficial for the degradation and transformation of pollutants, such as

nitrogen, phosphorous, and aromatic compounds (Hesselsoe *et al*., 2009). According to Figure 4.14 (panel C) *Xanthomonadales*, *Pseudomonadales*, and *Alteromonadales* are the major orders of *Gammaproteobacteria*. Płaza *et al.*, (2008) reported that *Pseudomonas* and *Xanthomonas* tolerate high concentrations of the hydrocarbons and have a high degradation capability. *Rhizobiales* were the main order of *Alphaproteobacteria* with the total abundances of 15.12%. *Rhizobia* are soil, Gram-negative bacteria with diverse functions which among them able to establish an N_2-fixing symbiosis is unique (Erlacher *et al*., 2015).

4.2.4. Comparison of CPBB performance with systems reported in the literature

The advantage of using circulating packed bed bioreactor was investigated earlier (Huang *et al*., 2012; D'Souza *et al*., 2014; Gunawan *et al*., 2014) which their characteristics of high oxygen transfer rate, high biomass hold-up, and mixing were noticeable. To use these merits, CPBB was operated to remove commercial NAs from contaminated water. The obtained results showed that increasing the NA concentration (50 to 200 mg NA L^{-1} (35 to 140 mg TOC L^{-1})) and loading rate (1.0 to 874.0 mg TOC L^{-1} h^{-1}) led to higher removal rate, while removal percentage was declined and then stabilized. The maximum removal rate of 128.0, 321.7, 430.2, and 630.0 mg TOC L^{-1} h^{-1} and removal percentages of 76.3, 87.6, 89.1, and 82.5% were realized at influent NA concentrations of 50, 100, 150, and 200 mg NA L^{-1}, respectively. This bioreactor could reduce the toxicity of NAs contaminated water which was influenced by the loading rate and influent concentration. At influent concentration of 100 to 200 mg NA L^{-1}, IC_{50} of 7.6 and 4.0% improved to 78.1 and 68.5% at the lowest loading rates and 41.7 and 36.5% at the highest loading rates, respectively.

To study the biodegradation of OSPW, researchers have evaluated various bioreactors using surrogates NAs, commercial NA mixtures, OSPW organic extracts, and real OSPW matrices. It should be noted that efficiency of bioreactors to remove commercial or surrogate NAs should not be translated into its performance for real OSPWs as they have more complex and recalcitrant compounds, and direct comparison of different bioreactors capabilities may not reveal all the details. Nevertheless, in order to have an efficient bioreactor for OSPW biodegradation, it has to easily function to eliminate surrogate or commercial NAs. Therefore, comparison of various studies is challenging. It becomes even harder as analytical methods may also affect the effluent removal efficiency of different bioreactors. Moreover, biodegradation

efficacy was reported in different ways including removal rate, removal percentage, and half-lives.

To better scrutinize how the developed system was able to remove the NAs, a comparison with the other flow reactors has been made and the results are presented in Table 4.8. First, as it can be seen there is not any other work that covers a wide range of operating conditions including concentration and loading rate; and most of them only focused on specific conditions. Second, the current work is among the few studies that all aspects of NAs removal including the fate of individual NAs, toxicity and microbial community have been considered. Third, as it can be seen from Table 4.8 and elaborated subsequently, compared to other bioreactors, CPBBs could remove influents at higher rates with high percentages. However, for better comparison, it is needed to run them with OSPW.

McKenzie *et al.* (2014) reported that immobilized cell reactors have the potential to improve NA removal rates. In their study, continuous operation of immobilized soil/sediment bioreactor (ISBR) with OSPW led to 38.4 % NA removal (removal rate: 2.3 mg NA L^{-1} d^{-1}) at a residence time of 161 h. The bioreactor was operated for 120 days at different organic and hydraulic loading rates. In another study, a fluidized bed biofilm reactor (FBBR) with granular activated carbon (GAC) as support media was used (Islam *et al*., 2014). The combined GAC adsorption and biodegradation process removed 38% of chemical oxygen demand (COD), 46% of an acid-extractable fraction (AEF) and 84% of classical NAs under optimized operational conditions. Using ozonated OSPW assisted them to reach higher removals. Huang *et al.* (2015,2017) found that the treatment of OSPW in a fixed film activated sludge bioreactor (IFAS) operated at a residence time of 48 h led to removal of 12.1% of the acid extractable fraction (AEF) and 43.1% of the parent NAs in OSPW. In two related works, Shi *et al.* (2015, 2017) utilized a moving bed biofilm bioreactor (MBBR) for OSPW treatment. According to their findings, 18.3% of AEF and 34.8% of NAs could be treated at a residence time of 48 h, while increasing residence time to 96 h did not have a major effect on the removal efficiency. (Zhang *et al.*, 2018) studied the effect of adjusting reactor HRT and nitrogen concentration in the performance of a MBR treating raw OSPW. HRT adjustment resulted in NA removal in the range of 33–50% for the treatment of ozonated OSPW whereas NA removal for raw OSPW only fluctuated between 27% and 38%.

Table 4.8 Comparison of CPBB performance with other systems reported in the literature

	Influent	HRT (h)	Influent Concentration (mg L^{-1})				Loading Rate (mg L^{-1} h^{-1})				Removal Rate (mg L^{-1} h^{-1})				Removal Percentage (%)				Toxicity change (IC_{50})
			COD	AEF	TOC	NA	COD	AEF	TOC	NA	COD	AEF	TOC	NA	COD	AEF	TOC	NA	
1	Ozonated OSPW	12.0	194.0	-	-	11.6	-	-	-	1.0	-	-	-	0.5	-	-	-	50.0	-
	Raw OSPW	12.0	223.0	-	-	55.3	-	-	-	4.6	-	-	-	1.8	-	-	-	38.0	-
2	Ozonated OSPW	48.0	180.2	88.6	-	9.5	3.8	1.8	-	0.2	1.9	0.3	-	0.1	51.0	15.0	-	47.5	46.3 to 54.1
	Raw OSPW	48.0	193.2	60.5	-	25.1	4.0	1.3	-	0.5	1.7	0.2	-	0.2	43.0	12.1	-	43.1	28.5 to 41.8
3	Ozonated OSPW	48.0	235.6	65.0	-	6.4	4.9	1.4	-	0.1	2.8	0.3	-	0.1	58.0	19.7	-	34.1	19.7%
	Raw OSPW	48.0	252.0	88.7	-	19.8	5.3	1.8	-	0.4	3.2	0.3	-	0.1	60.7	18.3	-	34.8	18.3%
4	Ozonated OSPW	2.5	150.0	68.0	-	1.5	60.0	27.2	-	0.6	36.0	23.4	-	0.6	60.0	86.0	-	99.5	-
	Raw OSPW	2.5	250.0	15.0	-	42.6	100.0	6.0	-	17.0	38.0	2.8	-	14.3	38.0	46.0	-	84.0	-
5	Raw OSPW	160.8	-	-	-	49	-	-	-	0.3	-	-	-	0.1	-	-	-	38.4	-
6	Commercial NAs	0.2-4.1	-	-	35.0-140.0	50.0-200.0	-	-	1.0-874.0	-	-	-	5.0-630.0	-	-	-	57.0-90.0	-	30.0-80.0%

1- Membrane Bioreactor (Zhang *et al*., 2018) 2- Integrated fixed-film activated sludge (Huang *et al*., 2017) 3- Fluidized Bed Bioreactor (Islam *et al.*, 2014)

4- Moving Bed Bioreactor (Shi *et al*., 2017) 5- Immobilized Soil/Sediment Bioreactor (McKenzie *et al*., 2014) 6- Present work

5. CONCLUSIONS AND RECOMMENDATIONS

5.1. Conclusions

In the present study, aerobic biodegradation of a commercial mixture of naphthenic acids was carried out in batch and circulating packed bed bioreactors. Although a number of studies have reported on different aspects of NAs biodegradation, most of them have ignored considering engineering parameters like concentration, loading rate, residence time, and temperature on the biodegradation. As a part of earlier works, it is shown that in circulating packed-bed bioreactor (CPBB), the diffusional limitations are reduced significantly, and efficient mass transfer can be achieved. The use of high porosity, high surface area packing in the riser of the bioreactor permits high biomass hold-up, while high air flow rates and resulting shear forces reduce or eliminate the plugging by biomass. So that in the current study, a wide range of concentrations at different temperatures and loading rates were investigated to find the impacts of these variables on the performance of CPBB and or removal of NAs from contaminated waters. As commercial NAs encompass a complex mixture of NAs, they are good surrogates of OSPW NAs and obtained results of this study can be used to simulate an efficient system for OSPW reclamation.

As the first objective of this study, batch biodegradation of 50, 100, 150 and 200 mg NA L^{-1} of commercial NAs in batch bioreactors demonstrated the potential of microbial activity for bioremediation of naphthenic acids.

- After 10 days about 75-80% of the initial total carbon concentration was removed.
- Biodegradation rate is a function of initial concentration where the highest rate of 0.78 mg TOC L^{-1} h^{-1} achieved at the highest initial concentration of 200 mg NA L^{-1} (140±4 mg TOC L^{-1}).
- Compositional study of commercial NAs by GC–MS showed that after incubation the percentage of NAs in the range of C_5-C_{13} dropped to 17% (from 29) and the percentage of NAs with Z=0 declined from 18.1 to 9.6% which were the highest among the other groups.

Therefore, the most biodegradable naphthenic acids were those with lower carbon and Z numbers.

- The obtained results of biodegradation at different temperatures showed that the biodegradation rate is not relying on temperature variation in the range of 20-35 ℃.

The second objective was to evaluate the performance of continuous flow CPBBs in treatment of NAs contaminated waters by investigating the effects of loading rate of NAs on their residual concentration, removal percentage, and removal rate through varying influent substrate(s) concentrations and influent flow rates.

- In all the influent concentrations, an increase of loading rate increased its residual TOC concentration and removal rate. Removal percentage, however, decreased and reached a minimum value, then had a negligible variation.
- At various influent initial concentration of 50, 100, 150 and 200 mg NA L^{-1}, the highest removal percentage of 76.3, 87.6, 89.1, 82.5% occurred at the lowest loading rates. It means that providing higher residence time led to higher NAs removals.
- At various influent initial concentration of 50, 100, 150 and 200 mg NA L^{-1}, the highest removal rate of 128.0, 321.7, 430.2, and 630 mg TOC L^{-1} h^{-1} evaluated at the highest loading rates. It shows that as removal rate comprises two opposing factors, residence time had major influence on the obtained removal rates.
- In terms of the distribution of individual NAs, low molecular weight acyclic NAs had higher removal levels. On the other hand, NAs with 13-22 carbon and Z= -4, -6 were the most recalcitrant compounds which removal of them may need higher residence time or integrating with other methods like ozonation.
- The toxicity analysis of treated effluents also confirmed they are less toxic toward *A.salina* as their survival percentage after 4 hours of exposure at the highest loading rate increased to 80, 46, 44, and 31% at various initial concentration. The Microtox results further indicated that the developed biofilm demonstrated the detoxification potential of NAs contaminated water where at influent concentration of 100 to 200 mg NA L^{-1}, 7.6 and 4.0% IC_{50} increased to 41.7 and 36.5% at the highest loading rates.

The results of the present study indicate that biodegradation of commercial naphthenic acid is influenced by the environmental conditions such as temperature, the concentration of naphthenic acid, loading rate and that maintaining the condition of biodegradation at the optimal

level could lead to enhancement of biodegradation. This study also helps with the understanding of the principles governing the biodegradation of OSPW NAs.

5.2. Recommendations for future work

The work presented in this thesis indicated that commercial naphthenic acids can be treated effectively in batch bioreactors and by running the CPBB in continuous operation. Comparison of experimental results indicated that the biodegradation rate of NAs can be significantly enhanced by varying operating conditions and using a well-designed bioreactor. However, further research in certain specific areas, as listed below, are required for more effective treatment and practical applications in the treatment of oil sand tailings, which are comprised of a very complex mixture of NAs.

In this study, biodegradation of naphthenic acids was investigated under aerobic conditions. There is little information about NAs biodegradation under an anaerobic condition, for example under denitrifying conditions. Further evaluation of the anaerobic process would be beneficial, especially that it could provide a basis for understanding the potential for *in*-situ bioremediation of oil sands naphthenic acid or in anoxic bioreactors where technical challenges and cost associated with aeration can be eliminated.

Additional research needs to be carried out using OSPW, which has a more complex structure. The results will confirm the potential of CPBBs for treatment of NAs and toxicity reduction of OSPW. Furthermore, using more accurate characterization methods will help with better elucidation of individual NAs.

In addition, using extensive experimental data generated in the batch system, a comprehensive kinetic expression for biodegradation of commercial NAs can be developed. The developed biokinetic expressions can be used in mathematical modeling and simulation of the biodegradation process in the CPBBs. The resulting information then will have beneficial applications in the design, scale-up, and control of the biodegradation process.

Finally, the study of process parameters such as the circulation rates, air flow rates, pressure drop, liquid holdup, and initial residence time for early stages of microbial activities is recommended for future work. Modeling of CPBB both from hydrodynamic and biokinetics points of view would be another interesting work which can be coupled with biokinetic models

to reliably predict removal of NAs in CPBBs. It would be also of interest to examine the effect of other operating factors, such as temperature, pH, and salinity on the biodegradation rate.

REFERENCES

Afzal, A., Chelme-Ayala, P., Drzewicz, P., Martin, J. W., & Gamal El-Din, M. (2015). Effects of Ozone and Ozone/Hydrogen Peroxide on the Degradation of Model and Real Oil-Sands-Process-Affected-Water Naphthenic Acids. Ozone: Science and Engineering, 37(1), 45–54. https://doi.org/10.1080/01919512.2014.967835

Ajaero, C., McMartin, D. W., Peru, K. M., Bailey, J., Haakensen, M., Friesen, Headley, J. V. (2017). Fourier Transform Ion Cyclotron Resonance Mass Spectrometry Characterization of Athabasca Oil Sand Process-Affected Waters Incubated in the Presence of Wetland Plants. Energy and Fuels, 31(2), 1731–1740. https://doi.org/10.1021/acs.energyfuels.6b02643

Allen, E. W. (2008). Process water treatment in Canada's oil sands industry: I. Target pollutants and treatment objectives. Journal of Environmental Engineering and Science, 7(2), 123–138. https://doi.org/10.1139/S07-038

Alpatova, A., Kim, E. S., Dong, S., Sun, N., Chelme-Ayala, P., & Gamal El-Din, M. (2014). Treatment of oil sands process-affected water with ceramic ultrafiltration membrane: Effects of operating conditions on membrane performance. Separation and Purification Technology, 122, 170–182. https://doi.org/10.1016/j.seppur.2013.11.005

Anhaecke, P., Persoone, G., Claus, C., & Sorgeloos, P. (1981). Proposal for a Short-Term Toxicity Test with Artemia Nauplii.

Azad, F. S., Abedi, J., & Iranmanesh, S. (2013). Removal of naphthenic acids using adsorption process and the effect of the addition of salt. Journal of Environmental Science and Health, Part A, 48(13), 1649–1654. https://doi.org/10.1080/10934529.2013.815457

Barrow, M. P., Headley, J. V., Peru, K. M., & Derrick, P. J. (2004). Fourier transform ion cyclotron resonance mass spectrometry of principal components in oilsands naphthenic acids. Journal of Chromatography A, 1058(1–2), 51–59. https://doi.org/10.1016/j.chroma.2004.08.082

Bartlett, A. J., Frank, R. A., Gillis, P. L., Parrott, J. L., Marentette, J. R., Brown, Hewitt, L. M.

(2017). Toxicity of naphthenic acids to invertebrates: Extracts from oil sands process-affected water versus commercial mixtures. Environmental Pollution, 227, 271–279. https://doi.org/10.1016/j.envpol.2017.04.056

Benally, C., Li, M., & Gamal El-Din, M. (2018). The effect of carboxyl multiwalled carbon nanotubes content on the structure and performance of polysulfone membranes for oil sands process-affected water treatment. Separation and Purification Technology, 199(December 2017), 170–181. https://doi.org/10.1016/j.seppur.2018.01.030

Bertheussen, A., Simon, S., & Sjöblom, J. (2018). Equilibrium Partitioning of Naphthenic Acid Mixture , Part 1 : Commercial Naphthenic Acid Mixture. Energy and Fuels, 32, 7519–7538. https://doi.org/10.1021/acs.energyfuels.8b01494

Biryukova, O. V., Fedorak, P. M., & Quideau, S. A. (2007). Biodegradation of naphthenic acids by rhizosphere microorganisms. Chemosphere, 67(10), 2058–2064. https://doi.org/10.1016/j.chemosphere.2006.11.063

Brown, L. D., & Ulrich, A. C. (2015). Oil sands naphthenic acids: A review of properties, measurement, and treatment. Chemosphere, 127, 276–290. https://doi.org/10.1016/j.chemosphere.2015.02.003

Chan, P. Y., Gamal El-Din, M., & Bolton, J. R. (2012). A solar-driven UV/Chlorine advanced oxidation process. Water Research, 46(17), 5672–5682. https://doi.org/10.1016/j.watres.2012.07.047

Clemente, J. S., & Fedorak, P. M. (2005). A review of the occurrence, analyses, toxicity, and biodegradation of naphthenic acids. Chemosphere, 60(5), 585–600. https://doi.org/10.1016/j.chemosphere.2005.02.065

Clemente, J. S., Mackinnon, M. D., & Fedorak, P. M. (2004). Aerobic Biodegradation of Two Commercial Naphthenic Acids Preparations. Environmental Science and Technology, 38(4), 1009–1016. https://doi.org/10.1021/es030543j

Clemente, J. S., Prasad, N. G. ., MacKinnon, M. D., & Fedorak, P. M. (2003). A statistical comparison of naphthenic acids characterized by gas chromatography–mass spectrometry. Chemosphere, 50(10), 1265–1274. https://doi.org/10.1016/S0045-6535(02)00763-4

D'Souza, L., Sami, Y., Nemati, M., & Headley, J. (2014). Continuous Co-biodegradation of Linear and Cyclic Naphthenic Acids in Circulating Packed-Bed Bioreactors. Environmental Progress & Sustainable Energy, 33(3), 835–843. https://doi.org/10.1002/ep

Del Rio, L. F., Hadwin, A. K. M., Pinto, L. J., MacKinnon, M. D., & Moore, M. M. (2006). Degradation of naphthenic acids by sediment micro-organisms. Journal of Applied Microbiology, 101(5), 1049–1061. https://doi.org/10.1111/j.1365-2672.2006.03005.x

Deriszadeh, A., Harding, T. G., & Husein, M. M. (2009). Improved MEUF removal of naphthenic acids from produced water. Journal of Membrane Science, 326(1), 161–167. https://doi.org/10.1016/j.memsci.2008.09.047

Dias, H. P., Pereira, T. M. C., Vanini, G., Dixini, P. V., Celante, V. G., Castro, Romão, W. (2014). Monitoring the degradation and the corrosion of naphthenic acids by electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry and atomic force microscopy. Fuel, 126, 85–95. https://doi.org/10.1016/j.fuel.2014.02.031

Erlacher, A., Soh, J., Grube, M., Cardinale, M., Sensen, C. W., Berg, G., & Cernava, T. (2015). Rhizobiales as functional and endosymbiontic members in the lichen symbiosis of Lobaria pulmonaria L. Frontiers in Microbiology, 6(February), 1–10. https://doi.org/10.3389/fmicb.2015.00053

Folwell, B. D., McGenity, T. J., Price, A., Johnson, R. J., & Whitby, C. (2016). Exploring the capacity for anaerobic biodegradation of polycyclic aromatic hydrocarbons and naphthenic acids by microbes from oil-sands-process-affected waters. International Biodeterioration and Biodegradation, 108, 214–221. https://doi.org/10.1016/j.ibiod.2014.12.016

Gamal El-Din, M., Fu, H., Wang, N., Chelme-Ayala, P., Pérez-Estrada, L., Drzewicz, P., Smith, D. W. (2011). Naphthenic acids speciation and removal during petroleum-coke adsorption and ozonation of oil sands process-affected water. Science of the Total Environment, 409(23), 5119–5125. https://doi.org/10.1016/j.scitotenv.2011.08.033

Godia, F., & Sola, C. (2005). Fluidized-bed bioreactors. Biotechnology Progress, 11(5), 479–497. https://doi.org/10.1021/bp00035a001

Gunawan, Y., Nemati, M., & Dalai, A. (2014). Biodegradation of a surrogate naphthenic acid

under denitrifying conditions. Water Research, 51, 11–24. https://doi.org/10.1016/j.watres.2013.12.016

Han, X., Scott, A. C., Fedorak, P. M., Bataineh, M., & Martin, J. W. (2008). Influence of molecular structure on the biodegradability of naphthenic acids. Environmental Science & Technology, 42(4), 1290–1295. https://doi.org/10.1021/es702220c

Headley, J. V., Peru, K. M., Tanapat, S., & Putz, G. (2002). Biodegradation Kinetics of Geometric Isomers of Model Naphthenic Acids in Athabasca River Water. Canadian Water Resources Journal, 27(1), 25–42. https://doi.org/10.4296/cwrj2701025

Headley, J. V., Du, J. L., Peru, K. M., & McMartin, D. W. (2009). Electrospray ionization mass spectrometry of the photodegradation of naphthenic acids mixtures irradiated with titanium dioxide. Journal of Environmental Science and Health - Part A Toxic/Hazardous Substances and Environmental Engineering, 44(6), 591–597. https://doi.org/10.1080/10934520902784625

Herman, D. C., Fedorak, P. M., MacKinnon, M. D., & Costerton, J. W. (1994). Biodegradation of naphthenic acids by microbial populations indigenous to oil sands tailings. Canadian Journal of Microbiology, 40(6), 467–477. https://doi.org/10.1139/m94-076

Hesselsoe, M., Füreder, S., Schloter, M., Bodrossy, L., Iversen, N., Roslev, P., … Loy, A. (2009). Isotope array analysis of Rhodocyclales uncovers functional redundancy and versatility in an activated sludge. ISME Journal, 3(12), 1349–1364. https://doi.org/10.1038/ismej.2009.78

Hindle, R., Noestheden, M., Peru, K., & Headley, J. (2013). Quantitative analysis of naphthenic acids in water by liquid chromatography-accurate mass time-of-flight mass spectrometry. Journal of Chromatography A, 1286, 166–174. https://doi.org/10.1016/j.chroma.2013.02.082

Holowenko, F. M., MacKinnon, M. D., & Fedorak, P. M. (2001). Naphthenic acids and surrogate naphthenic acids in methanogenic microcosms. Water Research, 35(11), 2595–2606. https://doi.org/10.1016/S0043-1354(00)00558-3

Holowenko, F. M., MacKinnon, M. D., & Fedorak, P. M. (2002). Characterization of naphthenic

acids in oil sands wastewaters by gas chromatography-mass spectrometry. Water Research, 36(11), 2843–2855. https://doi.org/10.1016/S0043-1354(01)00492-4

Huang, C., Shi, Y., Gamal El-Din, M., & Liu, Y. (2015). Treatment of oil sands process-affectedwater (OSPW) using ozonation combined with integrated fixed-film activated sludge (IFAS). Chemical Engineering Journal, 85, 167–176. https://doi.org/10.1016/j.cej.2016.11.151

Huang, J., Nemati, M., Hill, G., & Headley, J. (2012). Batch and continuous biodegradation of three model naphthenic acids in a circulating packed-bed bioreactor. Journal of Hazardous Materials, 201–202, 132–140. https://doi.org/10.1016/j.jhazmat.2011.11.052

Husein, M. M., Deriszadeh, A., & Harding, T. G. (2011). Experimental and modeling study of MEUF removal of naphthenic acids. Desalination, 273(2–3), 352–358. https://doi.org/10.1016/j.desal.2011.01.046

Hwang, G., Dong, T., Islam, M. S., Sheng, Z., Pérez-Estrada, L. A., Liu, Y., & Gamal El-Din, M. (2013). The impacts of ozonation on oil sands process-affected water biodegradability and biofilm formation characteristics in bioreactors. Bioresource Technology, 130, 269–277. https://doi.org/10.1016/j.biortech.2012.12.005

Iorhemen, O. T., Hamza, R. A., & Tay, J. H. (2016). Membrane bioreactor (Mbr) technology for wastewater treatment and reclamation: Membrane fouling. Membranes, 6(2), 13–16. https://doi.org/10.3390/membranes6020033

Islam, M. S., Dong, T., McPhedran, K. N., Sheng, Z., Zhang, Y., Liu, Y., & Gamal El-Din, M. (2014). Impact of ozonation pre-treatment of oil sands process-affected water on the operational performance of a GAC-fluidized bed biofilm reactor. Biodegradation, 25(6), 811–823. https://doi.org/10.1007/s10532-014-9701-6

Islam, M. S., Dong, T., Sheng, Z., Zhang, Y., Liu, Y., & Gamal El-Din, M. (2014). Microbial community structure and operational performance of a fluidized bed biofilm reactor treating oil sands process-affected water. International Biodeterioration and Biodegradation, 91, 111–118. https://doi.org/10.1016/j.ibiod.2014.03.017

John V. Headley, Kerry M. Peru, M. P. B. (2009). Mass spectrometric characterization of

naphthenic acids in environmental samples: a review. Mass Spectrometry Reviews, 28(12), 121–134. https://doi.org/10.1002/mas.20185

Kadic, E., & Heindel, T. J. (2014). An Introduction to Bioreactor Hydrodynamics and Gas-Liquid Mass Transfer. John Wiley & Sons. https://doi.org/10.1007/bfb0048441

Kannel, P. R., & Gan, T. Y. (2012). Naphthenic acids degradation and toxicity mitigation in tailings wastewater systems and aquatic environments: A review. Journal of Environmental Science and Health - Part A Toxic/Hazardous Substances and Environmental Engineering, 47(1), 1–21. https://doi.org/10.1080/10934529.2012.629574

Kim, E. S., Liu, Y., & Gamal El-Din, M. (2013). An in-situ integrated system of carbon nanotubes nanocomposite membrane for oil sands process-affected water treatment. Journal of Membrane Science, 429, 418–417. https://doi.org/10.1016/j.memsci.2012.11.077

Labrada, V., & Nemati, M. (2018). Biodegradation of surrogate naphthenic acids and electricity generation in microbial fuel cells : bioelectrochemical and microbial characterizations. Bioprocess and Biosystems Engineering, 0(0), 0. https://doi.org/10.1007/s00449-018-1989-x

Lari, E., Steinkey, D., Morandi, G., Rasmussen, J. B., Giesy, J. P., & Pyle, G. G. (2017). Oil sands process-affected water impairs feeding by Daphnia magna. Chemosphere, 175, 465–472. https://doi.org/10.1016/j.chemosphere.2017.02.088

Leshuk, T., de Oliveira Livera, D., Peru, K. M., Headley, J. V., Vijayaraghavan, S., Wong, T., & Gu, F. (2016). Photocatalytic degradation kinetics of naphthenic acids in oil sands process-affected water: Multifactorial determination of significant factors. Chemosphere, 165, 10–17. https://doi.org/10.1016/j.chemosphere.2016.08.115

Li, C., Fu, L., Stafford, J., Belosevic, M., & Gamal El-Din, M. (2017). The toxicity of oil sands process-affected water (OSPW): A critical review. Science of the Total Environment, 601–602, 1785–1802. https://doi.org/10.1016/j.scitotenv.2017.06.024

Liang, X., Zhu, X., & Butler, E. C. (2011). Comparison of four advanced oxidation processes for the removal of naphthenic acids from model oil sands process water. Journal of Hazardous Materials, 190(1–3), 168–176. https://doi.org/10.1016/j.jhazmat.2011.03.022

Liu, J., Wang, L., Tang, J., & Ma, J. (2016). Photocatalytic degradation of commercially sourced naphthenic acids by TiO 2 -graphene composite nanomaterial, 149, 328–335.

Lo, C. C., Brownlee, B. G., & Bunce, N. J. (2006). Mass spectrometric and toxicological assays of Athabasca oil sands naphthenic acids. Water Research, 40(4), 655–664. https://doi.org/10.1016/j.watres.2005.12.008

Lu, Q., Yan, B., Xie, L., Huang, J., Liu, Y., & Zeng, H. (2016). A two-step flocculation process on oil sands tailings treatment using oppositely charged polymer flocculants. Science of the Total Environment, 565, 369–375. https://doi.org/10.1016/j.scitotenv.2016.04.192

Mahaffey, A., & Dubé, M. (2017). Review of the composition and toxicity of oil sands process-affected water. Environmental Reviews, 25(1), 97–114. https://doi.org/10.1139/er-2015-0060

Martin, J. W., Barri, T., Han, X., Fedorak, P. M., El-Din, M. G., Perez, L., Jiang, J. T. (2010). Ozonation of oil sands process-affected water accelerates microbial bioremediation. Environmental Science and Technology, 44(21), 8350–8356. https://doi.org/10.1021/es101556z

McKenzie, N., Yue, S., Liu, X., Ramsay, B. A., & Ramsay, J. A. (2014). Biodegradation of naphthenic acids in oils sands process waters in an immobilized soil/sediment bioreactor. Chemosphere, 109, 164–172. https://doi.org/10.1016/j.chemosphere.2014.02.001

Merlin, M., Guigard, S. E., & Fedorak, P. M. (2007). Detecting naphthenic acids in waters by gas chromatography-mass spectrometry. Journal of Chromatography A, 1140(1–2), 225–229. https://doi.org/10.1016/j.chroma.2006.11.089

Mishra, S., Meda, V., Dalai, A. K., McMartin, D. W., Headley, J. V., & Peru, K. M. (2010). Photocatalysis of Naphthenic Acids in Water. Journal of Water Resource and Protection, 2(7), 644–650. https://doi.org/10.4236/jwarp.2010.27074

Misiti, T. M., Tezel, U., Tandukar, M., & Pavlostathis, S. G. (2013). Aerobic biotransformation potential of a commercial mixture of naphthenic acids. Water Research, 47(15), 5520–5534. https://doi.org/10.1016/j.watres.2013.06.032

Misiti, T., Tandukar, M., Tezel, U., & Pavlostathis, S. G. (2013). Inhibition and

biotransformation potential of naphthenic acids under different electron accepting conditions. Water Research, 47(1), 406–418. https://doi.org/10.1016/j.watres.2012.10.019

National Energy Board. (2017). Canada's Energy Future 2017, 90. Retrieved from https://www.neb-one.gc.ca/nrg/ntgrtd/ftr/2017/pblctn-eng.html

Natural Resources Canada. (2016). Energy Fact Book 2016 – 2017, 1–132. Retrieved from https://www.nrcan.gc.ca/sites/www.nrcan.gc.ca/files/energy/pdf/EnergyFactBook_2016_17_En.pdf

Natural Resources Defense Council. (2017). Alberta's tailings ponds, 1–8. Retrieved from https://environmentaldefence.ca/wp-content/uploads/2017/06/EDC-and-NRDC-One-trillion-litres-of-toxic-waste-and-growing-Albertas-tailings-ponds-June-2017.pdf

Nemati, M., & Webb, C. (2011). Immobilized Cell Bioreactors. Comprehensive Biotechnology, Second Edition (Second Edition, Vol. 2). Elsevier B.V. https://doi.org/10.1016/B978-0-08-088504-9.00100-8

Niasar, H. S., Li, H., Kasanneni, T. V. R., Ray, M. B., & Xu, C. C. (2016). Surface amination of activated carbon and petroleum coke for the removal of naphthenic acids and treatment of oil sands process-affected water (OSPW). Chemical Engineering Journal, 293, 189–199. https://doi.org/10.1016/j.cej.2016.02.062

Paslawski, J., Headley, J., Hill, G., & Nemati, M. (2009). Biodegradation kinetics of trans-4-methyl-1-cyclohexane carboxylic acid. Biodegradation, 20(1), 125–133. https://doi.org/10.1007/s10532-008-9206-2

Paslawski, J., Nemati, M., Hill, G., & Headley, J. (2009). Biodegradation kinetics of trans-4-methyl-1-cyclohexane carboxylic acid in continuously stirred tank and immobilized cell bioreactors. Journal of Chemical Technology and Biotechnology, 84(7), 992–1000. https://doi.org/10.1002/jctb.2122

Pérez-Pantoja, D., Donoso, R., Agulló, L., Córdova, M., Seeger, M., Pieper, D. H., & González, B. (2012). Genomic analysis of the potential for aromatic compounds biodegradation in Burkholderiales. Environmental Microbiology, 14(5), 1091–1117. https://doi.org/10.1111/j.1462-2920.2011.02613.x

Płaza, G. A., Berry, C. J., Nałęcz-Jawecki, G., Jangid, K., Łukasik, K., & Brigmon, R. L. (2008). Reduction of Petroleum Hydrocarbons and Toxicity in Refinery Wastewater by Bioremediation. Bulletin of Environmental Contamination and Toxicology, 81(4), 329–333. https://doi.org/10.1007/s00128-008-9411-z

Pourrezaei, P., Alpatova, A., Chelme-Ayala, P., Perez-Estrada, L. A., Jensen-Fontaine, M., Le, X. C., & Gamal El-Din, M. (2014). Impact of petroleum coke characteristics on the adsorption of the organic fractions from oil sands process-affected water. International Journal of Environmental Science and Technology, 11(7), 2037–2050. https://doi.org/10.1007/s13762-013-0406-x

Pourrezaei, P., Drzewicz, P., Wang, Y., Gamal El-Din, M., Perez-Estrada, L. A., Martin, J. W., … Giesy, J. P. (2011). The impact of metallic coagulants on the removal of organic compounds from oil sands process-affected water. Environmental Science and Technology, 45(19), 8452–8459. https://doi.org/10.1021/es201498v

Quagraine, E. K., Peterson, H. G., & Headley, J. V. (2005). In Situ Bioremediation of Naphthenic Acids Contaminated Tailing Pond Waters in the Athabasca Oil Sands Region—Demonstrated Field Studies and Plausible Options: A Review. Journal of Environmental Science and Health, Part A, 40(3), 685–722. https://doi.org/10.1081/ESE-200046649

Quinlan, P. J., & Tam, K. C. (2015). Water treatment technologies for the remediation of naphthenic acids in oil sands process-affected water. Chemical Engineering Journal, 279, 696–714. https://doi.org/10.1016/j.cej.2015.05.062

Scott, A. C., MacKinnon, M. D., & Fedorak, P. M. (2005). Naphthenic acids in athabasca oil sands tailings waters are less biodegradable than commercial naphthenic acids. Environmental Science and Technology, 39(21), 8388–8394. https://doi.org/10.1021/es051003k

Scott, A. C., Young, R. F., & Fedorak, P. M. (2008). Comparison of GC-MS and FTIR methods for quantifying naphthenic acids in water samples. Chemosphere, 73(8), 1258–1264. https://doi.org/10.1016/j.chemosphere.2008.07.024

Scott, A. C., Zubot, W., MacKinnon, M. D., Smith, D. W., & Fedorak, P. M. (2008). Ozonation

of oil sands process water removes naphthenic acids and toxicity. Chemosphere, 71(1), 156–160. https://doi.org/10.1016/j.chemosphere.2007.10.051

Shang, D., Kim, M., Haberl, M., & Legzdins, A. (2013). Development of a rapid liquid chromatography tandem mass spectrometry method for screening of trace naphthenic acids in aqueous environments. Journal of Chromatography A, 1278, 98–107. https://doi.org/10.1016/j.chroma.2012.12.078

Shu, Z., Li, C., Belosevic, M., Bolton, J. R., & El-din, M. G. (2014). suplementry-Application of a Solar UV/Chlorine Advanced Oxidation Process to Oil Sands Process-A ff ected Water Remediation, 1–24. https://doi.org/10.1021/es5017558

Sohrabi, V., Ross, M. S., Martin, J. W., & Barker, J. F. (2013). Potential for in situ chemical oxidation of acid extractable organics in oil sands process affected groundwater. Chemosphere, 93(11), 2698–2703. https://doi.org/10.1016/j.chemosphere.2013.08.072

St.John, W. P., Rughani, J., Green, S. A., & Mcginnis, G. D. (1998). Analysis and characterization of naphthenic acids by gas chromatography – electron impact mass spectrometry of tert . - butyldimethylsilyl derivatives, 807, 241–251.

St John, W. P., Rughani, J., Green, S. A., & McGinnis, G. D. (1998). Analysis and characterization of naphthenic acids by gas chromatography–electron impact mass spectrometry of tert.- butyldimethylsilyl derivatives. Journal of Chromatography A, 807, 241–251. https://doi.org/10.1016/S0021-9673(98)00085-5

Thompson, D. K., Motta, F. L., & Soares, J. B. P. (2017). Investigation on the flocculation of oil sands mature fine tailings with alkoxysilanes. Minerals Engineering, 111(May), 90–99. https://doi.org/10.1016/j.mineng.2017.06.008

Toor, N. S., Franz, E. D., Fedorak, P. M., MacKinnon, M. D., & Liber, K. (2013). Degradation and aquatic toxicity of naphthenic acids in oil sands process-affected waters using simulated wetlands. Chemosphere, 90(2), 449–458. https://doi.org/10.1016/j.chemosphere.2012.07.059

Trujillo, M. E. (2016). Actinobacteria. eLS. John Wiley & Sons, Ltd: Chichester., (2016), 1–16. https://doi.org/10.1002/9780470015902.a0020366.pub2

Vaiopoulou, E., Misiti, T. M., & Pavlostathis, S. G. (2015). Removal and toxicity reduction of naphthenic acids by ozonation and combined ozonation-aerobic biodegradation. Bioresource Technology, 179, 339–347. https://doi.org/10.1016/j.biortech.2014.12.058

Vajihinejad, V., & Soares, J. B. P. (2018). Monitoring Polymer Flocculation in Oil Sands Tailings: A Population Balance Model Approach. Chemical Engineering Journal. https://doi.org/10.1016/j.cej.2018.04.039

Venkataganesh, B., Maiti, A., Bhattacharjee, S., & De, S. (2012). Electric field assisted cross flow micellar enhanced ultrafiltration for removal of naphthenic acid. Separation and Purification Technology, 98, 36–45. https://doi.org/10.1016/j.seppur.2012.06.017

Wang, C., Alpatova, A., McPhedran, K. N., & Gamal El-Din, M. (2015). Coagulation/flocculation process with polyaluminum chloride for the remediation of oil sands process-affected water: Performance and mechanism study. Journal of Environmental Management, 160, 254–262. https://doi.org/10.1016/j.jenvman.2015.06.025

Wang, C., Huang, R., Klamerth, N., Chelme-Ayala, P., & Gamal El-Din, M. (2016). Positive and negative electrospray ionization analyses of the organic fractions in raw and oxidized oil sands process-affected water. Chemosphere, 165, 239–247. https://doi.org/10.1016/j.chemosphere.2016.09.009

Wang, C., Klamerth, N., Huang, R., Elnakar, H., & Gamal El-Din, M. (2016). Oxidation of Oil Sands Process-Affected Water by Potassium Ferrate(VI). Environmental Science and Technology, 50(8), 4238–4247. https://doi.org/10.1021/acs.est.5b04829

Wang, S., & Zhong, J. (2007). Chapter 6 . Bioreactor Engineering. Science, 131–161. https://doi.org/http://dx.doi.org/10.1016/B978-044452114-9/50007-4

Warnock, J. N., & Bratch, K. (2005). Chapter 4 Packed Bed Bioreactors. Culture, 87–113.

Whitby, C. (2010). Microbial naphthenic Acid degradation. Advances in applied microbiology (1st ed., Vol. 70). Elsevier Inc. https://doi.org/10.1016/S0065-2164(10)70003-4

Wilde, M. J., & Rowland, S. J. (2018). Naphthenic acids in oil sands process waters: Identification by conversion of the acids or esters to hydrocarbons. Organic Geochemistry, 115, 188–196. https://doi.org/10.1016/j.orggeochem.2017.09.004

Xue, J., Huang, C., Zhang, Y., Liu, Y., & Gamal El-Din, M. (2018). Bioreactors for oil sands process-affected water (OSPW) treatment: A critical review. Science of the Total Environment, 627, 916–933. https://doi.org/10.1016/j.scitotenv.2018.01.292

Xue, J., Zhang, Y., Liu, Y., & El-din, M. G. (2016). Treatment of oil sands process-affected water (OSPW) using a membrane bioreactor with a submerged fl at-sheet ceramic micro fi ltration membrane. Water Research, 88, 1–11. https://doi.org/10.1016/j.watres.2015.09.051

Yergeau, E., Lawrence, J. R., Sanschagrin, S., Waiser, M. J., Korber, D. R., & Greer, C. W. (2012). Next-generation sequencing of microbial communities in the athabasca river and its tributaries in relation to oil sands mining activities. Applied and Environmental Microbiology, 78(21), 7626–7637. https://doi.org/10.1128/AEM.02036-12

Yu, X., Lee, K., Ma, B., Asiedu, E., & Ulrich, A. C. (2018). Indigenous microorganisms residing in oil sands tailings biodegrade residual bitumen. Chemosphere, 209, 551–559. https://doi.org/10.1016/j.chemosphere.2018.06.126

Zhang, Y., Chelme-Ayala, P., Klamerth, N., & Gamal El-Din, M. (2017). Application of UV-irradiated Fe(III)-nitrilotriacetic acid (UV-Fe(III)NTA) and UV-NTA-Fenton systems to degrade model and natural occurring naphthenic acids. Chemosphere, 179, 359–366. https://doi.org/10.1016/j.chemosphere.2017.03.112

Zhang, Y., Islam, M. S., Liu, Y., McPhedran, K. N., & Gamal El-Din, M. (2015). Next-Generation Pyrosequencing Analysis of Microbial Biofilm Communities on Granular Activated Carbon in Treatment of Oil Sands Process-Affected Water. Applied and Environmental Microbiology, 81(12), 4037–4048. https://doi.org/10.1128/aem.04258-14

Zhang, Y., Xue, J., Liu, Y., & Gamal El-Din, M. (2018). The role of ozone pretreatment on optimization of membrane bioreactor for treatment of oil sands process-affected water. Journal of Hazardous Materials, 347, 470–477. https://doi.org/10.1016/j.jhazmat.2017.12.013

Zubot, W., MacKinnon, M. D., Chelme-Ayala, P., Smith, D. W., & Gamal El-Din, M. (2012). Petroleum coke adsorption as a water management option for oil sands process-affected water. Science of the Total Environment, 427–428, 364–372.

https://doi.org/10.1016/j.scitotenv.2012.04.024

APPENDIX A

Gas Chromatography-Mass Spectrometry analysis

The procedure to determine carbon and ring number based on measured m/z included the following steps:

First, the m/z values were rounded by setting the values between (x − 1).7 and (x).7 to x, since most atoms weigh more than the nominal mass. For example, both 237.7 m/z and 238.69 m/z would be rounded to 238 m/z. The n and Z were then determined using the matrix presented in Table A.1. The following assumptions were made to eliminate carbon number groups, and Z families that do not conform to the empirical formula, $C_nH_{2n+Z}O_2$, for NAs:

- if Z=0; at least one 5-carbon-member ring was present in the molecule
- there was one carbon atom available for the carboxyl group
- there was at least one carbon atom available for the alkyl R group,
- and structures with >3 rings (Zo_ 6) could be fused on more than two sides

The entries labeled ‘‘-’’, found predominately in the upper right corner of Table A.1, indicate cases in which there is an insufficient number of carbon or hydrogen atoms available to form the predicted number of ring structures indicated by the Z family designation. The masses shown are those of [M +57] ions because of the mass of the silyl group added through derivatization. The process of reading spectra masses, rounding, sorting, and generating plots has been programmed in a MATLAB subroutine.

Table A.1 Molecular weight of derivatized NAs [M+57] given by the formula $C_nH_{2n+Z}O_2$ distributed among carbon numbers and Z families

Carbon number	Z family						
	0	**-2**	**-4**	**-6**	**-8**	**-10**	**-12**
5	159	-	-	-	-	-	-
6	173	-	-	-	-	-	-
7	187	185	-	-	-	-	-
8	201	199	-	-	-	-	-
9	215	213	-	-	-	-	-
10	229	227	225	-	-	-	-
11	243	241	239	-	-	-	-
12	257	255	253	251	-	-	-
13	271	269	267	265	-	-	-
14	285	283	281	279	277	-	-
15	299	297	295	293	291	-	-
16	313	311	309	307	305	303	-
17	327	325	323	321	319	317	-
18	341	339	337	335	333	331	329
19	355	353	351	349	347	345	343
20	369	367	365	363	361	359	357
21	383	381	379	377	375	373	371
22	397	395	393	391	389	387	385
23	411	409	407	405	403	401	399
24	425	423	421	419	417	415	413
25	439	437	435	433	431	429	427
26	453	451	449	447	445	443	441
27	467	465	463	461	459	457	455
28	481	479	477	475	473	471	469
29	495	493	491	489	487	485	483
30	509	507	505	503	501	499	497
31	523	521	519	517	515	513	511
32	537	535	533	531	529	527	525
33	551	549	547	545	543	541	539

APPENDIX B

Sample Calculations

Hydraulic residence time (HRT), loading rate, removal rate, and removal percentage of NAs, for CPBB were calculated using the following procedures:

Hydraulic residence time = working volume of CPBB / flow rate

Loading rate = substrate influent concentration / hydraulic residence time

Removal rate = (influent concentration – residual concentration) / HRT

Removal percentage = (influent concentration – residual concentration) / influent concentration

APPENDIX C

Removal percentage and removal rate of CPBB effluents at different loading rates

Table C.1 Removal percentage and removal rate of NAs in CPBB at different loading rates (influent concentrations= 50 mg NA L^{-1})

Flowrate (ml h^{-1})	HRT (h)	Loading rate (mg TOC L^{-1} h^{-1})	Effluent TOC (mg TOC L^{-1})	Removal rate (mg TOC L^{-1} h^{-1})	Removal percentage (%)
10	45.00	0.8	8.3	0.6	76.3
45	10.00	3.5	8.3	2.7	76.3
60	7.50	4.7	8.4	3.6	76.0
75	6.00	5.8	8.5	4.4	75.7
90	5.00	7.0	8.6	5.3	75.4
100	4.50	7.8	9.1	5.8	74.0
150	3.00	11.7	9.3	8.6	73.4
315	1.11	31.5	13.2	19.6	62.3
520	0.67	52.0	14.5	30.5	58.6
850	0.41	85.0	14.9	48.8	57.4
1195	0.29	119.5	13.9	72.2	60.4
1650	0.21	165.0	12.5	106.1	64.3
2185	0.16	218.5	14.5	128.0	58.6

Table C.2 Removal percentage and removal rate of NAs in CPBB at different loading rates (influent concentrations= 100 mg NA L^{-1})

Flowrate (ml h^{-1})	HRT (h)	Loading rate (mg TOC L^{-1} h^{-1})	Effluent TOC (mg TOC L^{-1})	Removal rate (mg TOC L^{-1} h^{-1})	Removal percentage (%)
15	23.33	3.0	8.7	2.6	87.6
50	7.00	10.0	9.9	8.6	85.9
85	4.12	17.0	10	14.6	85.7
190	1.84	38.0	14.2	30.3	79.7
253	1.38	50.6	16.8	38.5	76.0
375	0.93	75.0	18.5	55.2	73.6
521	0.67	104.2	21.2	72.6	69.7
580	0.60	116.0	21.8	79.9	68.9
700	0.50	140.0	21.1	97.9	69.9
893	0.39	178.6	20.1	127.3	71.3
1219	0.29	243.8	19.5	175.8	72.1
1797	0.19	359.4	20.9	251.7	70.1
2278	0.15	455.6	20.6	321.7	70.6

Table C.3 Removal percentage and removal rate of NAs in CPBB at different loading rates (influent concentrations= 150 mg NA L^{-1})

Flowrate (ml h^{-1})	HRT (h)	Loading rate (mg TOC L^{-1} h^{-1})	Effluent TOC (mg TOC L^{-1})	Removal rate (mg TOC L^{-1} h^{-1})	Removal percentage (%)
85	4.12	25.5	11.4	22.7	89.1
253	1.38	75.9	15.2	64.9	85.5
521	0.67	156.3	17.7	129.9	83.1
893	0.39	267.9	22.6	210.0	78.4
1219	0.29	365.7	31.2	257.0	70.3
1725	0.20	517.5	37.1	334.8	64.7
2245	0.16	673.5	37.9	430.2	63.9

Table C.4 Removal percentage and removal rate of NAs in CPBB at different loading rates (influent concentrations = 200 mg NA L^{-1})

Flowrate (ml h^{-1})	**HRT (h)**	**Loading rate (mg TOC L^{-1} h^{-1})**	**Effluent TOC (mg TOC L^{-1})**	**Removal rate (mg TOC L^{-1} h^{-1})**	**Removal percentage (%)**
87	4.02	34.8	24.5	28.7	82.5
253	1.38	101.2	25.1	83.1	82.1
450	0.78	180.0	30.2	141.2	78.4
850	0.41	340.0	32.1	262.0	77.1
1195	0.29	478.0	32.1	368.4	77.1
1650	0.21	660.0	38.8	477.1	72.3
2185	0.16	874.0	39.1	630.0	72.1